Microbial synthesis of polymers: *Alcaligenes eutrophus* and the production of poly-β-3-hydroxybutyric acid (PHB).

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This thesis is submitted to the **CNAA** in part fullfillment of the requirements for the degree of **Doctor of Philosophy.**

The work undertaken in this study was in collaboration with **ICI Biological Products,** Billingham, Cleveland.

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Microbial Synthesis of Polymers :

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Lastly, indeterminate thanks are owed to the author's family, for their continued support and tolerance.

Declaration.

This is to certify that neither this thesis nor any part of it has been presented or is being concurrently submitted in candidature for any other degrees.

Candidate

Date: July 7th, 1989.

Certificate of Research.

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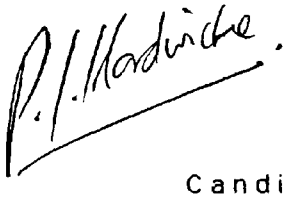

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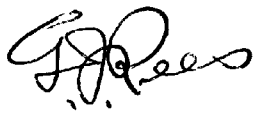


Director of studies

Date: July 7th, 1989

Summary - Microbial synthesis of polymers: *Alcaligenes eutrophus* and the production of poly-β-3-hydroxybutyric acid (PHB).

PHB formation in bacteria is a means to store reserves of energy and carbon. The location and discovery of **PHB** has been known since 1926. In the last 30 years, much attention has been given to it, due to the polymer's characteristics. It is a thermoplastic polyester, the first truly biodegradable plastic. The formation of **PHB** can be brought about by nutrient limitation, as the polymer is essentially a non-growth associated product in most bacteria (another species, **latus**, can produce it to excess of 60% dry weight, in exponential cells). Typically, nitrogen, phosphate and oxygen limitation are used to initiate storage.

This research set out to find a suitable production system. This first required a good growth medium. The maximum specfic growth rates of the research strain, **H/16 S301/C5**, and production strain, **H/16 S301/TRON**, were 0.73 and 0.61h^{-1}. This is believed to be faster than previously recorded rates. The scale-up from shake-flask to batch reactor was problematical; a 40-60% reduction in growth rate being typical. This was characterised as being due to Fe^{2+} limitation, due to precipitation with pH control.

During fed-batch experiments, of up to 50 hours, a maximum cell density of 12.4g/l was reached, and with up to 64% **PHB** formation. Continuous culture was not suitable for industrial production of **PHB**; biomass and **PHB** productivities were low, and at low dilution rates. However, it was suitable for producing **PHB**-free cells, to feed to a larger fed-batch production vessel. Two-stage batch and fed-batch production gave 15.65g/l cells, and up to 70% **PHB** production. Industrially, with an **ICI** CASE award session, fed-batch led to the production of 51g/l cells, with 80% copolymer. In a production run, a $50M^3$ vessel was operated, to produce 4 tonnes of polymer in 72 hours. Copolymer production is promoted by feeding organic acids. This leads to better polymer properties. One of the five experiments was run for 48 hours, reducing the running time by 24 hours. Enzyme determination of copolymer content was validated, and the current polymer extraction procedure was analysed, and found to be very satisfactory.

An industrial model was formulated, using a system where a chemostat would feed several large fed-batch production vessels. This gave a very good production, with an optimum figure of 2000-2250 tonnes p.a. If 316L stainless had been correctly stipulated in the model, then the optimum price for this level of production would be £11-12,000/tonne.

Chapter 1 - Introduction

At the beginning of this research, the proposed title was **"Microbial synthesis of polymers"**. This envisaged looking at a variety of microorganisms on different carbon sources, producing diverse polymers. After initial bench-scale work, scale-up to a 20l vessel would occur, along with analysis of downstream processing. One of the particularly interesting polymer candidates was **poly-β-3-hydroxybutyric acid (PHB)**. The alternative was a **polyacrylamide**. **PHB** was especially of interest due to it's biodegradability, and inherent environmental advantages. Along with the industrial link to **ICI Biological Products, Billingham** (who were to supply the cultures, and eventually a full collaboration) the project seemed very appealing.

Early discussions with **ICI** quickly established that **PHB** was the favoured polymer. Already the project had been narrowed down to one polymer, and to one or two organisms. This is a consideration which should not be underestimated by potential postgraduates and supervisors. If a specific part of the scenario is particularly interesting (providing it encompasses enough work), then the project may be steered towards this. Indeed, even well into the research, changes of direction are suitable, and are sometimes even necessary! Thus this project became **"The synthesis of poly-β-3-**

hydroxybutyric acid (PHB) by *Alcaligenes eutrophus*".

To refer to the original theme of microbial polymers, what would be considered appropriate? **Rees and Montgomery (formerly Underhill) (1986[1])**, the project supervisors, wrote a paper on this subject. Various polymeric substances could be considered, **PHB**, **polyphenylene**, **polyacrylamides** and **xanthan gum**. **ICI** have investigated both **PHB** and **polyphenylene** production. **Nitto**, in Japan, have investigated the synthesis of acrylamide monomers, with which to form polymers. Xanthan gum has been examined in the UK, produced by ***Xanthomonas campestris***. Which considerations have to be met in order to assess the potential production of microbial polymers?

Microbiological:

Screening techniques to isolate and identify suitable organisms, or improving on a process strain. This would proceed hand-in-hand with the next section.

Genetics:

Along with screening for organisms, genetic modification of current ones is necessary. Various mutagenic techniques are used to isolate different strains and mutants, some of which are metabolically

deficient in some way. These defective mutants help the wider understanding of the process, by examination of specific metabolic pathways and steps. Improvements to the process can be achieved, by engineering new strains from old ones.

Process Engineering:

Traditional chemical engineering practices have to be used to isolate, purify and present a finished product. Various downstream processing techniques would be used, dependent on where the polymer was located. The microbial polymers could be found in the cells, as an external coating, or even secreted into the medium. Examples of secreted polymers would include proteins. If the desired polymer was secreted, then extraction would be that much easier. Cellulose, formed by a small number of bacteria, is found as an external coating. In this case, the polymer has to be separated from the cell. In reality, the cells are dissolved with a strong alkali, and the recovered cellulose is then washed. Cellulose was not mentioned as a polymer of interest (for production), as vastly more research effort has been done on cellulose degradation. To produce microbial cellulose might have some applications, but the natural production of cellulose by plants is very extensive. Internal polymers present the biggest problem. In order to purify the polymer, it has to be first extracted. To extract the polymer, the cells

have to be damaged, or permeable to the extraction agent. If cells are damaged, then specific agents must be used, in order to protect the integrity of the polymer.

Economics:

To be economically suitable, various markets have to be examined. If the product is difficult to isolate, and costs are consequently high, then speciality high cost, low volume markets would be targetted.

The process must rely on a cheap raw material, in this case substrate. If the microbial production can proceed on a variety of cheap substrates, greater flexibility and economic protection is afforded. If one particular substrate becomes costly, then another can be substituted.

Process economics (on a finer scale) have to be satisfied. Does the polymer synthesis involve a good substrate to product conversion rate? Improvements to this (using new strains, for example) will obviously help matters and keep the process competitive.

Overall, enough resources must be recoverable, so that some may be used for further research. It is a foolish manufacturer, who gets a worldbeating process, and does not back it up with more research. In that case,

competitors would eventually catch-up and overhaul them.

The "bottom-line" is that toxic chemicals must be avoided throughout. The polymer produced must not be toxic or harmful in any way, to the environment of use, or the global environment. The process must be as safe as possible, in all respects.

Obviously, the above guidelines are very generalised, but give an idea of what must be considered. In the case of this research, **ICI Biological Products** were to be the industrial sponsors. Discussions with them eliminated polyacrylamides, as formation of acrylamides usually involves handling very toxic material. Xanthan gum production was already a well developed process. It was then decided to examine **PHB** in particular, using a single bacterial species.

What is **poly-β-3-hydroxybutyric acid (PHB)** and why is it so desirable to produce it? What properties does it exhibit, which warrant product formation?

PHB was first recognised, isolated and characterised by Lemoigne, in 1926 (**Dawes and Senior, (1973[2])**). In work (using ***Bacillus megaterium***) done up to 1940, it was proposed that **PHB** had an involvement with the sporulation process. Chemically, it is a lipid, the monomer of which is **3-hydroxybutyric acid (HB)**. **HB** is degraded by many

organisms, prokaryotic and eukaryotic, including humans. This natural degradability is of importance, and is described later on. Microbially, the lipid is produced as a reserve storage polymer. As such, it is one of several reserve materials, and bacteria either do not store any, store only one, or store several types, depending on the species. Physically, **PHB** is stored as single or several granules in the cell. It is present at about 10% of cell dry weight in a fully growing cell (during exponential growth). Under conditions of nutritional or physiological stress, individual cells swell-up disproportionately. They eventually reach levels of 95% **PHB**, when cells appear as membrane bound plastic ovals or spheres. Nuclear material eventually only occupies a very small space within the cell, crammed between the **PHB** and the cell membrane. **PHB** polymer granules are surrounded by a membrane, in which some of the synthesis and polymerase enzymes are sited. Consequently, early analysis of **PHB** granules (**Shively (1974**[3])) showed the presence of about 2% protein, trace quantities of phosphorous and a different lipid (the membrane). Individual **PHB** polymer granules are about 0.1-0.8μm in diameter (ø). The polymer itself is laid down into granules, which are crystalline in nature. In ***Bacillus cereus***, a central core is present, representing the virtually complete polymer chains. Further from the centre, the chains are in different stages of maturity and formation. Microscopically, **PHB** is very easy to distinguish, if not

actually see! Observation of **Gram** stained cells, containing high (>50% of cell dry weight) levels of **PHB**, show characteristically refractive areas. These correspond to polymer granules, which remain unstained. They are vividly seen, however, by staining with **Sudan Black**. Very good images can be seen using **Transmission Electron Microscopy (TEM)**, and can be useful in determining physiological changes, which occur during sporulation.

The precise nature of **PHB**, in respect to it's commercial interest, will be discussed later. Computer generated molecular models of pure polymers and copolymers are also shown.

Why is PHB formed?.

Any cells which have the ability to store energy, carbon, phosphate and ribonucleic acid (RNA) reserves for leaner times, will be better off. Such cells will be able to compete for otherwise hostile and barren environments. Competition between different genera and species would lead to a predilection of cells storing these reserves. The process of natural selection would ensure their advantage perpetuated. Of the most apparent types of reserve material, polysaccharides and lipids are valuable stores of energy and carbon. Polyphosphates, on the other hand, contribute energy only. RNA is a special, specific

metabolic reserve.

To compete for barren environments, how do cells use **PHB** to their advantage? If nutritional or physiological stresses are imposed on bacteria, some store polymers, some sporulate, and some do neither. In the case of nutritional stress, when a single nutrient (other than carbon) becomes limiting, exponential growth ceases. **PHB** formation can be initiated with limiting concentrations of O_2, N_2, K^+, Mg^{2+}, SO_4^{2-}, Fe^{2+}, Mn^{2+}, and phosphate (**Dawes and Senior (1973[2])**). With the cessation of growth, several things occur. As a specific nutrient is no longer available to promote cell division, cell metabolism changes. Observation of the **Pathway, Figure 1**, in this Chapter, shows the production of **PHB**, for example (**Senior and Dawes (1973[4])**, Oeding and Schlegel (1973[5]), and **Doi, et al (1987[6])**). As the requirements for vast energy intermediates (ATP) are not necessary, other than for maintenance energy, then certain compounds could accumulate. For the degradation of carbon substrates, such as glucose, pyruvic acid is formed in the **Embden Meyerhof (glycolytic) pathway**. This is converted into acetyl CoA, and supplies the **Kreb's Tricarboxylic acid (TCA) cycle**. If ATP formation is to be reduced, then this cycle must be slowed, to prevent ATP formation by the **Electron transport chain**. It is seen that the conversion of isocitric acid into α-Ketoglutarate, leads to the production of hydrogen ions and reduced **nicotinamide**

adenine dinucleotide phosphate (NAD(P)H). Further hydrogen ions are generated in three of the next four conversions. Thus to slow down ATP formation, one of two options is possible. Firstly, the metabolism of intermediates of the **TCA cycle**, or secondly, the formation of the **glyoxylic acid "shunt"**. If α-ketoglutarate, for example, is metabolised in order to maintain cell viability elsewhere, the cycle would slow or even stop. The glyoxylate cycle is used normally when not attacking primary substrates that lead to the formation of pyruvic acid. Instead, two molecules of acetyl CoA operate a "short-circuited" cycle, which leads to less hydrogen ions, and thus less ATP. The system is further adapted by removal of acetyl CoA to produce **PHB**. Initially, this may have been a method which just shunted the metabolism to slow down ATP formation. It is important to note, however, that if the electron transport chain is also slowed by less hydrogen ions, then NAD(P)H cannot be regenerated back to $NAD(P)^+$. This oxidation by the electron transport chain allows the TCA cycle to keep going normally. When the TCA cycle is not operating fully, NAD(P)H cannot be reoxidised properly. However, the formation of **PHB** reoxidises NAD(P)H in forming the coenzyme-linked monomer. If sufficient energy is provided to maintain the cells' viability (the so-called maintenance energy), without allowing growth, **PHB** can be used as a carbon and energy store. When nutritional conditions improve, cells which were

incapable of storing **PHB** would probably have perished. This would allow **PHB**-storers to grow with reduced competition. It should be noted how lactic acid and ethanol are included in the pathway, for **PHB** production. **Taylor and Anthony (1976[7])** were examining ***Pseudomonas* AMI**, and discovered **PHB** production during growth on ethanol and lactate. It is also notable, in addition, that **PHB** is not produced with any drain on ATP whatsoever. This is again an advantage over other reserve polymers, which utilise ATP. **PHB** is formed when available energy for maintenance is barely adequate, and not sufficient to promote other reserves.

PHB has also several other roles and uses for bacteria. Initially, **PHB** diverts the accumulation of acidic intermediates, and itself exerts no osmotic effect. Thus it is an inert, stable material. Other important roles for **PHB** are seen with specific bacteria. In the gram +ve spore forming ***Bacillus*** and ***Clostridium*** genera, **PHB** is associated with sporulation (**Emeruwa and Hawirko (1973[8])**. **PHB** granules are actually present in mature spores, although at low levels, and spore-less mutants cannot degrade **PHB**. Once exponential growth ceases, **PHB** is accumulated to a maximum level. In sporing strains, spore formation and maturation proceed at the expense of **PHB**. The polymer is obviously the "driving force" behind sporulation, in terms of supplying energy and carbon to produce spores.

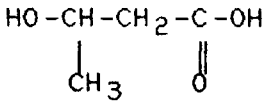
In the asporogenous strains, the lack of sporulation, and no **PHB** degradation, suggest a genetic link with the two processes. Presumably, the genetic material responsible for both was removed or damaged during mutation.

PHB also has a role in protecting oxidative damage of nitrogen fixing aerobes. ***Azotobacter*** is frequently found in the microaerobic rhizosphere, where O_2 concentrations are very low and it's **PHB** formation is stimulated. If the concentration of O_2 present is too high, growth and **PHB** formation are inhibited. Thus, in an environment where little oxygen is present, ***Azotobacter*** can survive periods of nutrient limitation (other than nitrogen). When such limitation is lifted, by replenishment of suitable nutrients, ***Azotobacter*** would grow again. By growing fast in low oxygen concentrations, it can override other survivors, growing slower. Thus, **PHB** affords ecological advantage, in this instance.

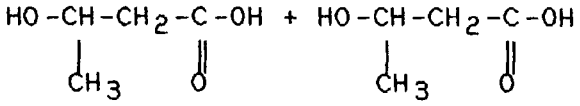
The distribution of **PHB** storing organisms is diverse, and is shown in **Table 1.** A second table is also shown in the **Appendix Section, Table A1.** The latter describes the distribution based on **"Bergey's determinative bacteriology", 1974.** The table in this chapter, corresponds to the updated **"Bergey's systematic bacteriology", 1984.** To gain a proper view, consult both, as the taxonomic position of several "old" genera is now

unsure (***Nitrobacter, Thiobacillus***, etc). This diversity is probably evidence of a common ancestoral prokaryotic cell, from which most bacteria have now developed. The acknowledgment of symbiotic bacteria, in the new **"Bergey"**, ties in well with ***Tetrahymena.*** This is the only known eukaryote capable of storing **PHB**. It is most likely that this protozoan ingested a **PHB** storing bacteria, which it has retained, according to the **"endosymbiosis theory"**. If the bacteria survived being used as food, then now it receives nutrients from the host. The protozoa now gains the advantage of being able to utilise stored **PHB**. Whether **PHB** is used at the expense of the endosymbiont, or not, is unclear. If one discounts the common ancestor idea, then the evolutionary advantage of **PHB** accumulation is powerfully seen.

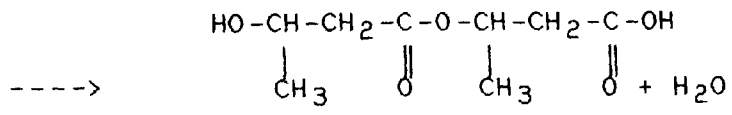
The structure of pure **PHB** is composed of linked **D(-)-3-hydroxybutyric acid** monomers, of the formula:



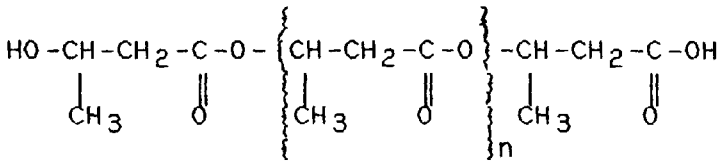
This polymerises :



With the loss of one molecule of H_2O (condensation)



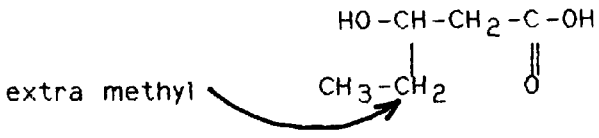
To form:



terminal group polymer chain terminal group

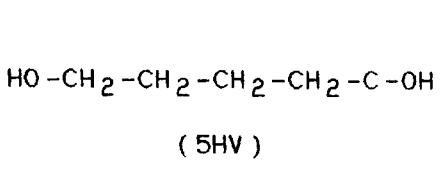
where n = the number of repeating monomer units found in the polymer.

Several copolymers have been found, similar to **PHB**. The most obvious is **poly(HB/HV)**, where **HV** is **D-3-hydroxyvaleric acid (HV)**. This is simply a methylated version of **HB**:

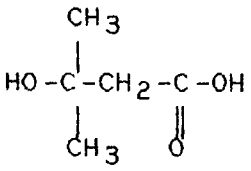


The copolymer is built up of random monomers of **HB** and **HV** in the chain. The structure and properties are consequently altered. This polymer is also made with varying mol% of **HV**, due to the carbon feed regime.

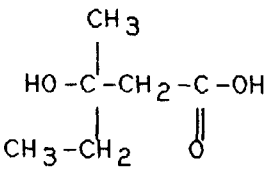
Another monomer is **D-5-hydroxyvaleric acid (5HV)**, where the monomer is an elongated chain, rather than branched. Two further monomers are **3-hydroxy isovaleric acid (3-hydroxy-3-methyl butanoic acid, HMB)** and **3-hydroxy isocaproic acid (3-hydroxy-3-methyl pentanoic acid, HMP)**. **HMB** is an isomer of **HV**, whereby the additional methyl group on **HB** is put onto the third carbon. **HMP** is a further methylated version of **HMB**, and is an isomer of 3-hydroxy hexanoic acid. **5HV, HMB and HMP** are shown:



(5HV)



(HMB)



(HMP)

Pure polymers of **HMB** and **HMP** were characterised by **Iwakura, et al (1971[9])**. These were synthetically made pure polymers, and have not been seen in bacteria. **Doi, et al (1987[10])** described the production of copolymers of **poly(HB/HV/5HV)**. All these variations of structure, on **PHB**, have important connotations. **Poly(HB/HV)** has a lower melting point than pure **PHB**, and is less brittle. The use of models to see what the polymer structures look like, is an important predictive tool. Using the **Chem-X** program (developed and distributed by Chemical Design Ltd., Oxford, England), run on the VAX mainframe computer at the **Polytechnic of Wales**, simulated structures were created.

These structures are:-

1-Pure **PHB**, containing eight monomer units, and two terminal groups.

2-Copolymer of **poly(HB/HV)**, containing alternately, nine monomer units of both **HV** and **HB**, with one terminal group of each.

3-Pure polymer of **HMB**, containing eight monomers and two terminal groups.

4-Pure polymer of **HMP**, containing eight monomers and two terminal groups.

5-Copolymer of **poly(HB/HV/5HV)**, containing eight monomer groups of each, alternately, with terminal groups of **HB** and **HV**, respectively.

These are shown at the end of the Chapter, **Figures 2-6.**

Using such a program, the models can be optimised, to predict the most thermodynamically stable structure. By combining different monomers, the polymer chain structure becomes apparent. This could be used to predict some of the polymers physical properties (such as whether the polymer could be drawn into a fibre, for example). The problem with the copolymers, as depicted, is that the monomers are arranged A-B-A-B-A-B or A-B-C-A-B-C-A-B-C, etc. In reality, the monomers would be inserted randomly, like A-A-B-B-B-A-C-C-B-C-C-C-C-A-A-A-B, etc. However, if this is accepted, the program can be used as

a powerful predictive tool for unusual structures, or even blends of two copolymers. Minimum energy profile graphs can be constructed, to give likely stable structures. Large-scale screening of bacteria, on a wide variety of substrates, would determine whether any other structures were likely. The full importance of altering pure polymer and copolymer structures, is that this affects the application of each polymer.

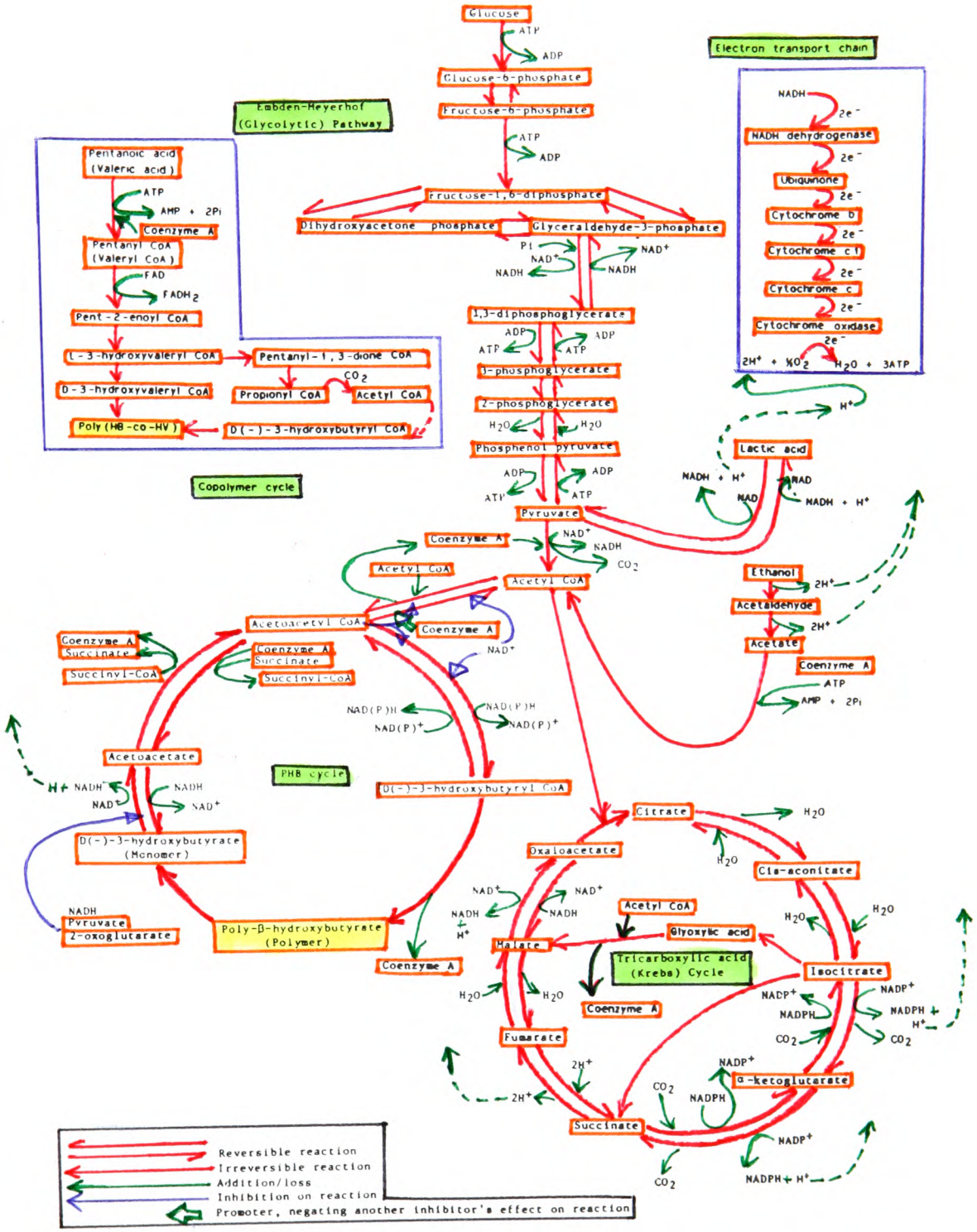
All of the previous considerations were merely academic, until the discovery that **PHB** and copolymers could be used as plastics. **PHB** is described as a thermoplastic polyester, which in physical characteristics is very similar to synthetic polyethylene. If **PHB** could be produced as cheaply as synthetic plastic, the market and environmental importance would be immeasureable. The first industrial application of **PHB**-containing cells is probably creditable to **Baptist and Werber, (1963[11])**. Here, whole cells (of ***Rhizobium***, containing 40-95% **PHB**) were compressed into sheets of a light brown plastic. Sometimes extracted **PHB** was added before heating and compression. The plastic formed was supposedly analagous to the production of plastic by the protein **casein**. At this time, however, the biodegradability of **PHB** was not realised. Subsequent research discovered **PHB** synthesis to be cyclical, and it could be broken down by bacteria, after production. Indeed, if cells are grown, allowed to

store **PHB**, and cultivated for too long, **PHB** is degraded due to cell autolysis. If nutrient limitation is lifted, from very high % **PHB**-containing cells, **PHB** is rapidly utilised. The degradation of **PHB** is perhaps most easily thought of as almost exclusively fungal. If **PHB** is buried, predominant attack is then due to extracellular enzymes, secreted by fungal mycelia. An important factor in the commercial development of **PHB**, is the degradation by animals, including humans. Humans possess the enzyme **3-hydroxybutyrate dehydrogenase** naturally. As **PHB** production will initially be low volume, high cost, medical applications can be targetted.

Historically, two groups are responsible for "championing" **PHB's** biodegradability. Peter Senior, formerly of **ICI Biological Products**, wrote (1984[12]) how **"I fondly believed I had got the world's first biodegradable, melt processable, oil-independent thermoplastic polyester in my hands"**. This inspirational statement arose after he buried a "poor solvent-cast film" of **PHB**, in the rose garden of **Hull University**, where he completed his PhD. The second group is headed by Robert Lafferty in Austria, who works closely with **Chemie Linz**. They developed **PHB** matrix tablets, to deliver drugs and agrochemicals. The chemicals are released over a fairly lengthy period of time (weeks), in a controlled, linear manner. **Table 2**, at the of the Chapter, lists some of the applications of **PHB** and copolymers. Paul Holmes,

again formerly of **ICI Biological Products**, did a great deal of work on **PHB** properties and applications. In an article he wrote (1985[13]), he describes several important considerations. **"The number of potential applications (for PHB) is almost infinite, and is limited only by our imagination"**. It is noted how **ICI** engineers, who discovered polyethylene in the 1930s, described how they thought the only application for this was in high-value electrical insulation for radar! **PHB**, must also not be underestimated. The company who markets **PHB**, and copolymers, must do so properly, and reap the benefit! If **PHB** can be produced at a very high tonnage level, then the greater the number of environmentally detrimental plastics that will suffer, the better! The author of this thesis awaits with considerable interest!

Figure 1.



Formation of PHB, showing connection to glycolysis and the tricarboxylic acid cycle

Table 1.

Occurence of PHB forming organisms:

Type	Genera
A. Kingdom ***Prokaryotae*** - **Cyanobacteria** - the blue/green bacteria.	various
B. Kingdom ***Prokaryotae*** - True bacteria (according to Bergey, 1984)	
2. Aerobic or microaerophilic, motile, helical or vibroid gram +ve bacteria.	**Aquaspirillum*, *Spirillum*, **Azospirillum*, **Oceanospirillum*
4. Gram -ve aerobic rods and cocci.	
Family 1 - ***Pseudomonadaceae***.	***Pseudomonas***, ***Zooglea***
Family 2 - ***Azotobacteraceae***.	Several species from all genera
Family 3 - ***Rhizobiaceae***.	***Rhizobium***, ****Bradyrhizobium***
Family 4 - ***Methylomonadaceae***.	***Methylococcus***, ***Methylobacterium*** and possibly others.
Family 5 - ***Halobacteriaceae***.	***Halobacterium***
Family 8 - ***Neisseriaceae***.	***Moraxella***, ***Acinetobacter***
Other genera: -	***Beijerinckia***, ***Derxia***, ***Alcaligenes*** ***Paracoccus***, ***Lampropedia***.
5. Facultatively anaerobic gram +ve rods Family 2 - ***Vibrionaceae***.	***Vibrio***, ***Photobacterium***
12. Gram +ve cocci.	***Micrococcus***
13. Endospore forming gram +ve rods and cocci.	***Bacillus***, ***Clostridium***
15. Irregular, non-spore formers, gram +ve rods.	***Actinomyces***
17. Nocardioforms.	***Nocardia***
C. Kingdom ***Eukaryotae*** subdivision: unicellular protists, microscopic protozoans.	***Tetrahymena*** (possibly due to endosymbiosis of a **PHB** forming organism, like those in Section 11 of the new Bergey manual

The sections of Bergey, 1974 which have apparently been "lost", include parts 1, 3, 4 and 12. These include bacteria such as ***Rhodopseudomonas***, ***Chromatium***, ***Sphaerotilus***, ***Caulobacter***, ***Nitrobacter***, and ***Thiobacillus***. Even the ***Streptomyces*** genus is apparently mentioned only "in passing". The taxonomic position of these organisms is now uncertain. The old classification system has been used in the Table in the **Appendix** Section, Table **A1**.

* - This signifies new genera described in Bergey, 1984, for the 1st time

Figure 2 - Pure polymer of PHB, containing eight monomers, and two terminal groups.

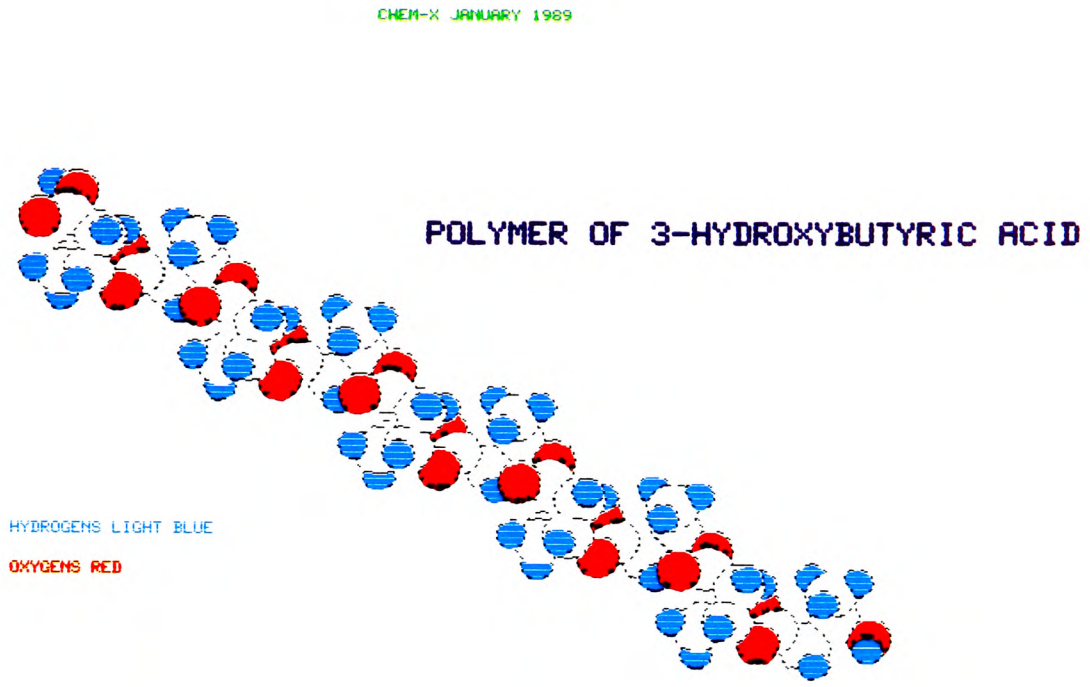


Figure 3 - Copolymer of poly(HB/HV), containing alternately, nine monomer units of both HV and HB, with one terminal group of each.

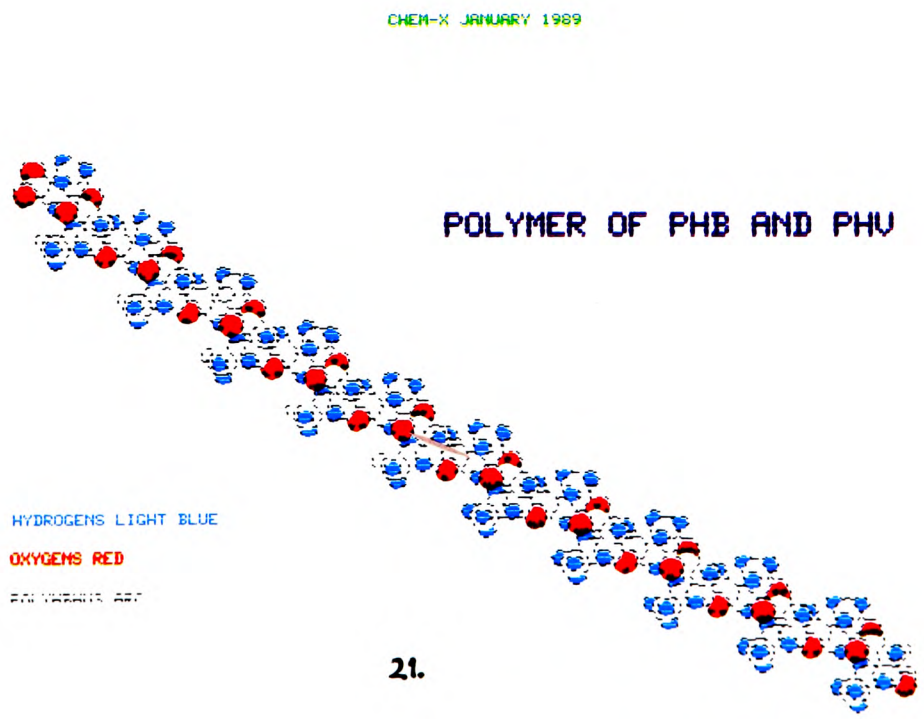


Figure 4 - Pure polymer of HMB, containing eight monomers and two terminal groups.

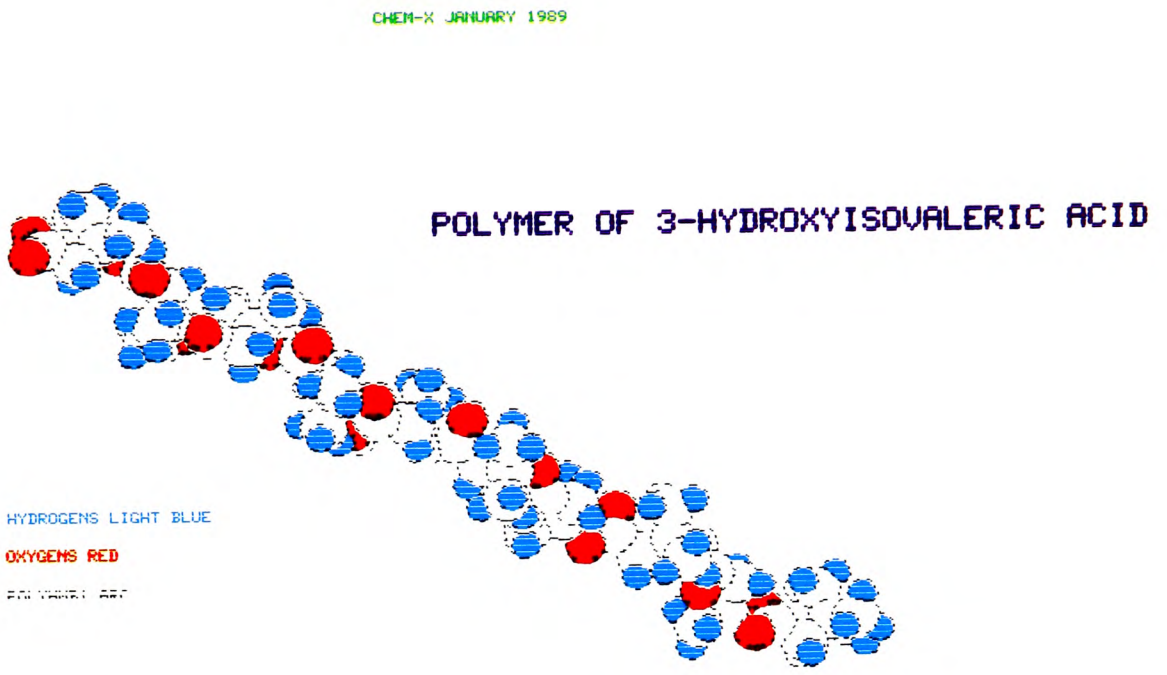


Figure 5 - Pure polymer of HMP, containing eight monomers and two terminal groups.

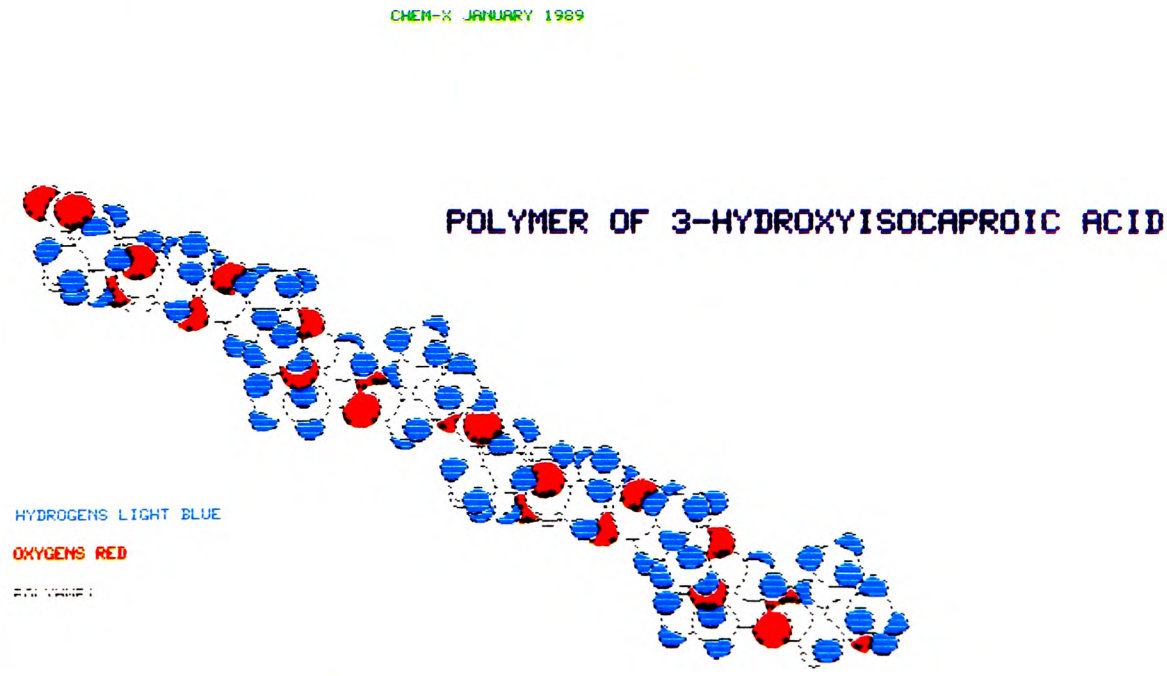


Figure 6 - Copolymer of poly(HB/HV/5HV), containing eight monomer groups of each, alternately, and two terminal groups of HB and HV, respectively.

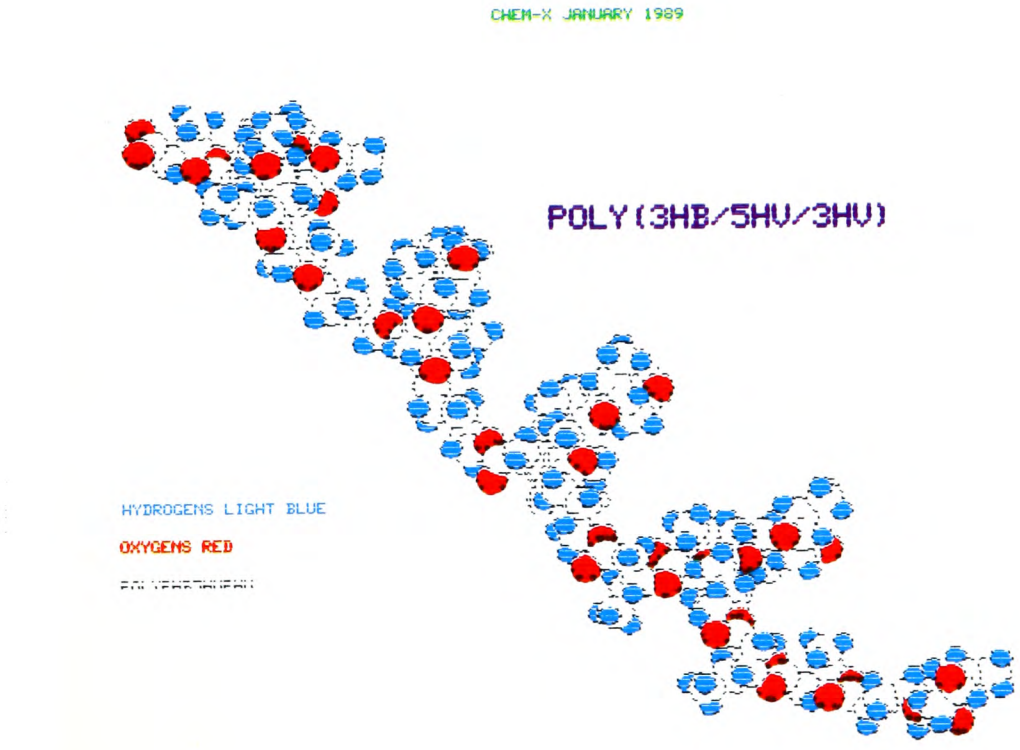


Table 2.

Table of likely markets for PHB (or copolymers such as "BIOPOL"):

Type of market	Application
1.High cost, low volume:	
(a)Medical:	-Biodegradable sutures, bone repair pins and plates. -Swabs and ligature clips.
(b)Pharmaceutical/agricultural. material delivery matrices.	-Pharmaceutical implantation; subcutaneous or intramuscular, controlled release of drugs over a lengthy period of time. -Agricultural application; plant protection chemicals: insecticides, herbicides, and also fertilisers.
2.Medium cost, Medium volume:	
(a)hygiene/disposable products.	-Nappies, feminine hygiene, adult incontinence packs and colostomy bags.
(b)Packaging/bottling.	-Food packing films, and bottles for foods and cosmetics.
(c)Electrical products.	-Piezoelectric films, for various electrical hardware.
(d)Various.	-Fine chemical synthesis. Polymers, copolymers and monomers used to produce synthetic chemicals, not produced in microbes. -Treatment films, for blood filtration and denitrification of water.
3.Low cost, high volume:	
(a)General plastics.	-Too many to list, of enormous economic value and importance.

The third section would only be viable, if very high production levels and low costs were sustainable. The conventional production of plastics, relying on oil, would have to be damaged by high oil prices, such as occurred during the oil crisis of the 1970s.

Chapter 2 - Literature Survey

To bring the story of **PHB** production up to date, it is necessary to go back to the polymer's first discovery in bacterial cells. The majority of early work was done in France, under the auspices of **Lemoigne** and fellow workers. This work was predominantly involved with the study of ***Bacillus megaterium*** and the process of sporulation. Lemoigne, et al, worked and published papers in this area from at least 1925 to as late as 1950. This time span would have coincided with the discovery of penicillin, and the scientific revolution created by antibiotics in the 1940s. In 1926-27, Lemoigne, et al, were able to show that several compounds (produced by hydrolysing bacterial cells) were derived from a polymer of **β-hydroxybutyrate**. This polymer was first characterised as having a melting point of 157°C. Compared to the pure polymer formed nowadays, this would represent either a low molecular weight or a slightly degraded polymer. It is likely that in hydrolysing the cells, the **PHB** also suffered degradation. In 1940, it was observed that **PHB** could have a physiological role in sporulation. The presence of **PHB** and lipid granules in other ***Bacillus*** species was reported in 1943. At this time, **PHB** was shown to be involved with cyst formation in ***Azotobacter chroococcum***. A year later, **PHB** was used as a means to classify the genus ***Bacillus***, and four taxonomic groups were created (either cells were **PHB** +ve

or -ve, and **acetoin** +ve or -ve). The discovery of ***Azotobacter chroococcum*** utilising and storing **PHB** was an important link to the majority of earliest work conducted in this country. This was done at **Hull University** with Professor E.A.Dawes, and will be discussed in some detail later. The early discoveries are made more remarkable by the fact that during the early 1940s, France was under German occupation, at the culmination of the Second World War. It is not clear whether Lemoigne, et al, actually worked in Paris at the time, or merely published their work there. Even if resident elsewhere, the events surrounding France at that time would have hindered them physically and mentally, be they resident in Paris or not. The early story of **PHB** was retained in Paris until 1955, when Tinelli demonstrated an actual role in the sporulation process. In 1958, a variety of workers started to look at the process of **PHB** accumulation in relation to bacterial physiology. Eventually work was done on the metabolism and enzymatics of formation. In 1960, the earliest patent for the industrial use of **PHB** containing cells was filed by the W.R.Grace company, New York. In 1976 a Swiss company patented the use of a class of solvents to extract **PHB** from within cells. From 1979-1987, both **ICI Biological Products** (in the UK) and the **University of Graz/ Chemie Linz** (in Austria) have filed patents relating to **PHB** processes. Now a detailed review of the history of the study of **PHB** will be told from 1958-1988 (a suitable

time-scale!), in distinct areas of research.

Areas of research concerning **PHB** formation and industrial biotechnological processes:

2.1 **PHB** metabolism and enzymic analysis pertinent to polymer production (1958-1985).

2.2 Occurrence of **PHB** in microbes (1958-1986).

2.3 **PHB** production, fermentation processes (1971-1987).

2.4 Conditions necessary to grow the relevant bacteria for **PHB** formation (1961-1984).

2.5 Physiological role of **PHB** formation (1967-1974).

2.6 **PHB** determination in cell material (1972-1986).

2.7 **PHB** recovery; extraction processes (1976-1987).

2.8 Biodegradable polymers (1983-1988).

2.9 Moulding and quality control/ assurance techniques (1960-1983).

2.10 Application and structure of **PHB** (1964-1987).

The survey is not exhaustive, but contains most of the salient papers and patents, especially the more recent ones.

2.1 **PHB** metabolism and enzymic analysis pertinent to polymer production.

Tinelli in 1955 was still continuing the work on **PHB** accumulation (with ***Bacillus megaterium***) in Paris, and

three years later the study of **PHB** left France. **Macrae and Wilkinson** (1958[14]) were also looking at **PHB** formation in ***Bacillus***. They published a paper in the **Journal of General Microbiology**, on the **PHB** metabolism in washed suspensions of ***Bacillus megaterium*** and ***Bacillus cereus***. They were able to demonstate that if glucose, pyruvate, and **3-hydroxybutyrate** (in addition to acetate) were fed to the bacteria, **PHB** storage was stimulated. This was done on an asporogeneous strain, to study the relationship of polymer storage and cell physiology, irrespective of sporulation.

Merrick and Doudoroff (1961[15]) examined the presence of polymer synthetase and depolymerases associated with the **PHB** granules. The granules are bound by a lipid membrane, in which the enzymes were situated. Various workers had attempted to characterise the percentage protein and non-**PHB** lipid in polymer granules. If these membrane associated proteins were deactivated, either by heat or chemically, then the degradation of granules was stopped. **Kominek and Halvorson** (1965[16]) looked at **PHBs** association with sporulation. By studying ***Bacillus cereus***, they were able to show (significantly as ***Bacillus megaterium*** was not similar) that **PHB** occured only if an acidic shift was created. As the cells grew, the pH reverted back to the original value, and **PHB** was used as a carbon and energy source. Sporulation then proceeded (although **PHB** was not essential for

sporulation). Other than infering **PHB** could be used as a source of energy and carbon, no specific role for **PHB** was put forward. **Griebel, et al** (1968[17]) continued the work of Merrick and Doudoroff on the molecular biology of **PHB** synthesis and degradation. These first four references were cited in the review by **Dawes and Senior** (1973[2]).

In the early 1960s, both Professors E.A.Dawes and H.G.Schlegel (of the **Universities of Hull**, UK, and **Göttingen**, FRG, respectively) were looking into reserve polymers in microbial systems. In this section of the **Literature Survey**, the next work was done by **Senior, et al**, under Professor **Dawes** (1972[18]), and **Senior and Dawes** (1973[4]). The first paper concerned the production of **PHB** by ***Azotobacter beijerinckii***, whilst under oxygen limitation. The use of a distinctly different bacterial system (***Azotobacter***) was a sign of the increasing interest in the study of **PHB**. **Senior, et al** (1972[18]), showed that **PHB** was formed, when cell growth and respiration had decreased the dissolved oxygen concentration (DOT) down to zero. This was with nitrogen limited batch cultures of ***Azotobacter***. To confirm whether **PHB** was formed as a result of oxygen or nitrogen limitation, chemostat experiments were initiated. This was necessary, as nitrogen limited cultures could still fix nitrogen from the aeration. The chemostat experiments proved conclusively that oxygen limitation was responsible for enhanced **PHB** storage. For an aerobic

organism, this seemed suprising; to have the **PHB** necessary to survive nutrient starvation, virtual anaerobic conditions were necessary! However, this protected the process of nitrogen fixation, which is oxygen sensitive. With a low oxygen concentration, the growth yield was higher. Due to **PHB** formation, an electron sink was available to reform nicotinamide adenine dinucleotide (phosphate) (NAD^+ and $NADP^+$, oxidised). Thus **PHB** formation is not solely to provide carbon and energy. **Senior and Dawes (1973[4])** looked at the regulation of **PHB** metabolism in ***Azotobacter beijerinckii***. Many enyzmes of the metabolism, both anabolic and catabolic, were studied and a pathway for **PHB** production was proposed. They noted the similarity with the system proposed by Schlegel's work on ***Alcaligenes eutrophus*** (formerly known as ***Hydrogenomonas eutropha***). The name of Peter Senior mentioned here, is a significant one in the British Industrial production of **PHB**. Under the guidance of Professor Dawes, he achieved his PhD in 1972, and later went on to work at **ICI Billingham** in the early 70s, leaving in the early 80s. Whilst at **ICI**, he initiated the work on industrial production of **PHB**.

At the same time as **Senior and Dawes (1973[4])** published their paper, **Oeding and Schlegel (1973[5])** published work on ***Alcaligenes eutrophus***. This also concerned the regulation of **PHB** metabolism. Indeed, the

two sets of workers were cooperating, in order to achieve a greater understanding of the subject as a whole. **Oeding and Schlegel** were trying to elucidate the molecular pathway of **PHB** synthesis, and proposed a cyclical scheme. The work showed that the production and degradation of **PHB**, in both bacteria, was essentially identical. Although members of the same taxonomic Section of **Bergey's Systematic Bacteriology, 1984**, they are members of separate families. It has been suggested that **PHB** synthesis is a mechanism for evolutionary advantage. It is also possible that, in view of the fact that many other unrelated organisms can store **PHB**, this is evidence of a common "probacteria" ancestor. Even the ***Tetrahymena*** protozoa can produce **PHB**, but this is probably a result of endosymbiosis of a **PHB** forming bacteria. This bacteria would now have lost its separate identity, but is probably like those listed in Section 11 of the new Bergey Manual (on endosymbionts). The possibility of a common ancestor is more likely than separate evolution of many bacteria, which all produced **PHB** in the same manner. This theme will be developed further in **Chapter 10**, the **Conclusions**.

The story moves back to the UK with **Taylor and Anthony (1976[7])**. They were examining the molecular pathways associated with the methylotroph ***Pseudomonas AM1***. This is again part of the same taxonomic section, but yet another family. The proposed pathway for this

organism fitted the other two organisms. They noted, however, that ***Hyphomicrobium***, also a **PHB** former, had a slightly different mechanism. This organism is even more widely taxonomically and genetically distinct from the other three, which might explain the difference.

Jackson and Dawes (1976[19]) again focus on ***Azotobacter beijerinckii***, in their work on the tricarboxylic acid cycle (TCA Cycle) and **PHB** metabolism. This study, of bacteria under O_2 or N_2 limitation, was more an academic one of the molecular pathways. **Ward, et al (1977[20])** (also part of the Hull group), used chemoheterotrophically grown ***Azotobacter***. Under N_2 and O_2 limitation, **PHB** metabolism was studied on ammonia grown cells. **Carter and Dawes (1979[21])** continued the work on **PHB** production under O_2 limitation. **Cook and Schlegel (1978[22])** studied the metabolite concentrations of ***Alcaligenes eutrophus***, with **PHB** +ve and -ve storing strains. When the cells were subject to a carbon and nitrogen-free environment, metabolites such as NAD^+ and adenosine triphosphate (ATP) were present in both. When CO_2 was fed to the cells, the mutant excreted pyruvate and malate, and accumulated fructose-6- phosphate, in marked contrast to the parent strain. Thus without the mechanism of forming **PHB**, the mutant was able to fix CO_2. When nitrogen was added, the cells resumed identical metabolic regimes. By knowing what cells do when unable to synthesise **PHB**, a picture of the purpose of **PHB**

accumulation emerges. Other than produce carbon and energy, it is a suitable "electron sink" for the reoxidation of NADH and NADPH. This is the case even during exponential growth, where **PHB** is present to about 10% of the dry weight.

Sonnleitner, et al (1979[23]) (another major group looking at **PHB** synthesis, at the **University of Graz**, Austria) characterised the formal kinetics of **PHB** formation in ***Alcaligenes eutrophus*** and ***Mycoplana rubra***. The kinetics were similar (although ***Mycoplana*** grew slower) in most respects. There was also the suggestion that too much oxygen inhibited the growth of ***Alcaligenes***. **Heinzle and Lafferty (1980[24])** put forward a kinetic model for **PHB** synthesis in ***Alcaligenes eutrophus***. This was the first work involving computer simulations and comparisons, which Elmar Heinzle and colleagues have developed to a high level nowadays. The other author, Robert Lafferty, worked with Professor Schlegel previously. He is responsible for work on **PHB** in Austria, in conjunction with the **Chemie Linz** company (which rivals **ICI** for the potential industrial production of **PHB**). The use of computer models and simulations, developed in this paper, are one of the most important biotechnological tools available for the industry in the future. They are widely used in teaching, with an obvious potential for research and production. Basic research can be formed into a model, which can be

predictive, to identify likely areas of interest.

Nur, et al (1982[25]), studied the effect of DOT on **PHB** and other molecular metabolites. They used ***Azospirillum brasiliense*** grown in continuous culture. The genus ***Azospirillum*** is a recently classified one in Bergey, 1984. This group, in Israel, found that microaerophilic conditions also allowed the production of **PHB** and certain necessary metabolites. Under high oxygen tension, **PHB** decreased and protein increased. This is a similar system employed in ***Azotobacter***, as elucidated by **Senior, et al (1972[18])**. This is further possible evidence of a "common ancestor" theory. **Nur, et al (1982[26])**, published further work on this organism, reiterating their earlier findings. They conclude that ***Azospirillum*** is very good at finding specific niches in the rhizosphere (the layer in the soil around the roots). **Siegel and Ollis (1984[27])** investigated the kinetics of another ***Alcaligenes eutrophus*** strain, in continuous culture. This also gave a better response, in terms of growth rate, under conditions of low oxygen tension. **PHB** was also encouraged, but still did not amount to more than 20% of cell dry weight. This has important connotations for the mass production (on an industrial-scale) of **PHB** formation. This will be made apparent due to the results of this thesis. **Braunegg and Bogensburger (1985[28])** examined a new species, ***Alcaligenes latus*** (strain **DSM 1123, NCIB 12189**). This was claimed to be a better

organism than ***Alcaligenes eutrophus***; able to synthesise **PHB** at a comparable rate and level, but induced faster in the growth cycle. It was claimed to be a more industrially useful organism.

2.2 Occurrence of PHB in microbes.

Historically, **PHB** had been detected in ***Bacillus*** species. The references cited in the first section (**1–19**) include a wide variety of organisms. It should be noted, however, that it is "microbes" and not "bacteria" solely. This is because **PHB** was found in a protozoan. In addition to these references, a major work was undertaken by **Stockdale, Ribbons and Dawes (1968[29])**. This paper lists the maximum percentage **PHB** accumulation for six ***Azotobacter*** species (and seven other strains), four species of ***Beijerinckia*** and one of ***Derxia***. Among these, ***Azotobacter beijerinckii*** **NCIB 9067** was the best, accumulating over 70% of cell dry weight as **PHB**. All the three genera have survived reclassification in the new Bergey Manual of 1984. The organisms were grown on nitrogen free medium (with one exception), which had 1 or 2% glucose as the carbon source. Through measurement of DOT, it was suggested here that oxygen might possibly be linked to **PHB** synthesis. This was later confirmed in the group's research in 1972.

Emeruwa and Hawirko (1973[8]) characterised **PHB**

formation in spore forming and asporogenous strains of ***Clostridium botulinum***. In the asporogenous mutant, **PHB** accumulated in a similar manner to the spore forming strain. However, instead of being utilised to provide a carbon and energy source for spore maturation, the asporogenous mutant continued to have a high content of **PHB**. In the spore forming strain, by the time that free spores were present, the percentage of **PHB** left in the cells was 25% of the maximum value. The spore itself had only 2% of its weight as **PHB**. This was the first report of the formation of **PHB** in a ***Clostridium*** species. **Kitase (1983[30])** reported the accumulation of **PHB** in a ***Vibrio*** species. **Tal and Okon (1985[31])** again cited **PHB** accumulation in ***Azospirillum brasiliense***. The ***Halobacterium*** genus was suggested as a novel possible biotechnological vehicle to produce **PHB**. **Fernandez-Castillo, et al (1986[32])** describe how the extreme halophile ***Halobacterium mediterranei***, grown with a 15% salt concentration, accumulated up to 45% cell dry weight as **PHB**. The novelty of the organism is due to it's halophilic nature, which, suggest the authors, enable "almost no sterile precautions" to be used. If the organism, grown at 37^{o}C, does not require much in the way of sterility, costs of production could be significantly lower. However, no mention is given to the density of culture produced. **Martin and Stewart (1986[33])** refute the suggestion that ***Acinetobacter*** is not able to synthesise **PHB**, with evidence of 3 strains which can. The

PHB synthetase enzymes were to be investigated, and the declaration was part of the proceedings of the Annual meeting of the American Society of Microbiology.

The occurrence of **PHB** in microbes is summarised into a Table, found in the **Introduction** (and another Table based on the old Bergey Classification of 1974 is found in the **Appendix Section**. The majority of information was culled from **Dawes and Senior (1973[2])**, the previous references and looking through Bergey's Manuals.

2.3 Polymer production and fermentation processes.

The first reference actually concerns the importance of forming copolymer based on **PHB**. The simplest copolymer consists of **hydroxybutyrate (HB)** and **hydroxyvalerate (HV)**, which is a methylated form of **HB**. The importance of **HV** monomers is that they increase the elasticity of the polymer. This would make the moulded plastic more suitable for industrial use. To incorporate **HV** into the polymer chain, the bacteria are fed propionic acid. The technique will be covered later. The initial reference by **Iwakura, et al (1971[9])** concerns the synthetic production of polymers. These were **poly-α-hydroxy- isovalerate** (3-hydroxy-3-methyl butanoic acid **HMB**) and **poly -α- hydroxyisocaproate** (3-hydroxy -3- methyl propanoic acid **HMP**). These structures are shown in the **Introduction**, and demonstrate the effect of methylated versions of the **HB**

and **HV** monomers on polymer structure. Microbially, **HMB** and **HMP** have not been formed. If they were, additional classes of polymers could be produced, and the application could be more widespread.

One of several early patents issued by **ICI** in the late 1970s and early 1980s, covers the manufacture of **PHB** by a methylotroph, ***Methylobacterium organophilum***. **Powell, Collinson and Richardson (1980[34])**, proposed the production of **PHB** by several strains of the bacterium. At the time, there were suitable quantities of methanol available, which could be used as a cheap carbon source. Mutant strains (from the normal parental strains) were developed, which could grow on methane, even after methanol incubation. The system employed was to grow the cells continuously, with a defined medium, and with no limitation. This allowed maximal (or just sub-maximal) growth. A second stage was operated, so that either nitrogen or phosphorus was limited. This resulted in a cell density of 35-55g/l, which was 50% **PHB**. The polymer was recovered by milling or shearing cells, followed by solvent extraction. They conclude that other methanol utilising strains of ***Pseudomonas rosea, Hyphomicrobium variable, Pseudomonas AM1*** (and other ***Methylobacterium organophilum*** strains) do not give sufficiently high quantities or qualities of **PHB**.

Hughes and Richardson (1982[35]), of **ICI**, put forward a

method producing **PHB** using ***Alcaligenes eutrophus.*** This was the same time as **Heinzle and Lafferty** were using the organism in Austria, and getting **Chemie Linz** interested industrially. Historically, Professor Schlegel studied **PHB** production with this organism in 1961, and Lafferty worked with Schlegel later on. This current patent mentioned that five species of the ***Alcaligenes*** genus produced **PHB**, namely ***faecalis, ruhlandii, latus, aquamarinus and eutrophus.*** **ICI** proposed the use of ***Alcaligenes eutrophus H16***, as this allowed a high accumulation of **PHB**. Additionally, it had a higher carbon conversion rate than previously described species. Of particular note, was the discovery of strain **H16 S301/C5.** This was used as the major bacteria of study in this thesis. It seems likely that as **ICI** had now become involved with using ***Alcaligenes eutrophus***, the Austrians decided to look at ***Alcaligenes latus.*** Indeed, they still work with this organism. This was probably a move to protect their interests, as **ICI** had developed and patented superior strains. An important breakthrough was getting a glucose utilising mutant. These differed from the parent strain, which could not utilise this carbon source. By growing the bacteria in continuous culture, **ICI** hoped to encourage good cell density and a good level of polymer storage.

Initially, batch cultures were run, resulting in a cell density of 45g/l, of which 50% was **PHB** (after 70

hours incubation). These had a carbon conversion rate of about 55-57% (where 100% conversion would mean 1g of carbon source, such as glucose, would give rise to 1g of **PHB**). Continuous culture gave cell densities of 5-6g/l, with 30-47% **PHB**, and a carbon conversion of about 70% . This reaction was conducted in a 5l vessel, under a dilution rate of $0.1h^{-1}$. By running one of the continuous culture vessels into a second, twice as large vessel, a 2-week culture was sustained. This provided 11g/l cells, with 70% **PHB**, in the second 10l vessel (7l nominal volume), operated at a dilution rate of $0.05h^{-1}$. The combination of the vessels led to an overall carbon conversion rate of 58%. The figure for the second vessel was considerably lower, being only 45%. **ICI** were able, however, to produce an effluent of 350ml/hour, at 11g/l cells, with 8g/l **PHB**. Thus **ICI** were able to produce 2.8g of **PHB**/hour, for 2 weeks, giving a total production of 0.94kg. Using this system, they could theoretically produce 21.6kg/year (46-week operational year). Whilst not a large production, it is an interesting figure. All these systems used nitrogen limitation to stimulate **PHB** production. If carbon limitation was imposed, cell density halved, and the **PHB** was less than half that found in exponentially growing cells. If we scaled-up 21.6kg/year, produced in a 10l vessel, to a $50M^3$ vessel, as operated in the scheme proposed in **Chapter 10**, then there would be about 120 tonnes of **PHB** produced. This compares with 480 tonnes/year, using a single $50M^3$

vessel. With the scheme proposed in **Chapter 10**, there would be 18 $50M^3$ vessels, producing nearly 9000 tonnes of **PHB**/year! As a direct comparison, to produce roughly 100 tonnes/year, it would require only one $10M^3$ vessel. Thus a two-stage continuous/fed-batch system, as proposed, would be better, especially at even larger tonnages. What is also interesting is the dilution rates; 0.1 and $0.05h^{-1}$. This low level would allow **PHB** to be produced, but both cell growth and density would be restricted. By reference to **Chapter 9**, this becomes apparent.

Holmes, Wright and Collins (1983[36]) characterised the development of the current **ICI** "BIOPOL" plastic range. Paul Holmes, in particular, realised the physical properties of **PHB** were not ideal, it crystallised too quickly, for example. This crystallinity detrimentally affected the moulding process. A ***Nocardia*** species, grown on butane, produced a copolymer of **poly-HB** and 3-hydroxy-2-butenoate. This was very similar to **HB**, but lacked one hydrogen atom. **3-hydroxyvalerate (HV)** monomers were also noted in this patent. Propionic acid was suggested as a means of creating **HV** monomers in the polymer chain. Various experimental regimes were created, in which ***Alcaligenes eutrophus NCIB 11599*** was fed propionic acid. **HV** was incorporated into the polymer to about 30%, which itself accounted for up to 70% of the dry weight. ***Nocardia salmonicolor*** was also used, and when propionic acid was present, 10% of the dry weight

consisted of a 72% **HV** copolymer. This patent describes a vast quantity of fermentations, using a variety of organic acids or derivatives. The only other monomer formed (other than **HB**) was **HV**, in any significant quantity. This then formed the basis of producing the currently created "BIOPOL" range. Depending on the particular application, a variety of copolymers can be created, differing in their **HV** content. The variation in the polymer is created by the feeding regime (by the mixture of the propionic acid/glucose feed). Details of the structure of **HB** and **HV** monomers is found in the **Introduction**, which helps to describe the reasons behind copolymer characteristics.

Richardson (1984[37]) described a process for the recycle of non-**PHB** cell material. ***Alcaligenes eutrophus NCIB 11599*** was grown to produce 50% of the cell dry weight as **PHB** (using glucose as the carbon source to generate pure polymer). After solvent extraction, the polymer-free cell material was hydrolysed in 6M HCl. The neutralised hydrolysate was used to feed the bacteria again. This was a novel process which, due to the currently used extraction process, was not to see the light of day.

Braunegg and Korneti (1984[38]) described a process to produce **PHB** from a pseudomonad. ***Pseudomonas 2F*** was isolated from soil around Graz, Austria, and selected for

experimentation. The significant point to the work was the observation of a novel process coined "carbon overcompensation". The culture was grown to produce **PHB**, but carbon became limiting. When glucose and ammonia were reconstituted, **PHB** was accumulated at a very fast rate initially. This was claimed to be similar to the process of "phosphate overcompensation" in phosphate limited yeast and ***Aerobacter aerogenes.***

As mentioned previously, one of the key workers in the industrial production of **PHB** was Peter Senior. He worked for Professor Dawes in Hull, up to the early 1970s. The next reference, **Senior (1984[12])**, provides a glimpse at the **ICI** process. It is notable that he describes how "In 1976 **ICI** entered the fray, with a very small team of one". Although published in 1984, the review is actually of around 1981, and even then he predicted that **ICI** might have a marketable product in 1983. **ICI** in actual fact do not have a marketable product even now, in late 1988. **Rees and Montgomery (formerly Underhill) (1986[1])** wrote a paper on the microbial synthesis of polymers. This was originally the loose title of this author's research, and they are the project supervisors. The paper introduces the concept of biopolymers, and assesses both production and likely economics. Economics can be a good way of predicting progress in a field. As costs alter around the globe, suddenly one process can become unproductive, and another

steps in. The production of **PHB** by **ICI** is mentioned, and is most easily compared to polypropylene production. Economically, there is no competition. However, by application to specific markets, **PHB** could well have a use in future markets. If economics permitted, then a market for nearly 1.4 million tonnes would exist (based on the US production for 1978). Many of **PHBs** properties are mentioned, which have been summarised in the **Introduction**. This paper gave the present author some feel for the area in which the research would proceed, before narrowing down to **PHB** in particular.

Lafferty and Braunegg (1985[39,40]) proposed a continuous two-stage production of **PHB**, using ***Alcaligenes latus*** **DSM 1123**. Under a dilution rate of $0.4h^{-1}$, the organism produced polymer to 71% of the cell dry weight. The cell density was 16.5g/l, and was produced in a 15l vessel, with an outflow of 99.1g of biomass/hour. This fed a second vessel, of 25l, which was operated at a dilution rate of $0.3h^{-1}$. **PHB** was produced to 79%, with a 35.5g/l cell density, and a polymer production rate of 210g/hour! Operated on a yearly (46-week) scale, this would amount to 1.6 tonnes. Therefore to produce about 100 tonnes of **PHB**, their system would require a second stage vessel of $2M^3$. This is a significant increase on the **ICI** method, and even betters the scheme proposed in **Chapter 10** five times.

Several contentious proposals were put forward under these patents. The authors describe initially the problems of using ***Alcaligenes eutrophus***, and then proceed to describe how to overcome them using ***Alcaligenes latus***. The **ICI** process (using ***Alcaligenes eutrophus***) has to be maintained at a low dilution rate, and thus to produce **PHB**, the growth is compromised. Apparently the operating temperature of 30-34^{o}C is also problematical; in a large vessel this would require excessive cooling capacity. **ICI** had attained a glucose utilising mutant, but both glucose and fructose are relatively expensive sources of carbon. This patent examines ***Alcaligenes latus***, and concludes that this would be a more economic system to produce **PHB**. It can be grown at high dilution rates, up to 0.4h^{-1}, and **PHB** accumulation proceeds even during exponential growth, to a high level. Indeed, it is stated that growth associated **PHB** formation, leads to cells having no less than 60% **PHB** at all times. Additionally, ***latus*** can grow on a very varied choice of carbon source. This would make the process of **PHB** less sensitive to economic pressure. If any particular carbon source (sucrose, for example) suddenly became very expensive, then a suitable alternative could be rapidly substituted. The fact that **PHB** is growth associated, means that the residence times are also shorter; experiments last only 30-40 hours. This contrasts to the normal residence times of 50-70 hours. ***Alcaligenes latus*** is also able to be grown at higher

temperatures, in the range 36-42°C, usually at 37°C. From experience at **ICI**, the author of this thesis has grown ***Alcaligenes eutrophus*** at 37 and even 40°C. Thus this advantage is negated. The dissolved oxygen content is now very low, less than 50%. Typically in this system it was 30 and 10%, in the small and large vessels respectively. These levels were enough to keep the system going, suggesting that ***Alcaligenes latus*** is less aerotolerant. ***Alcaligenes eutrophus***, by means of comparison, has a maximum growth rate when the air was at 60% air saturation. Of note in the medium, was the inclusion of cobalt or nickel. These are normally requirements of autotrophically grown ***Alcaligenes eutrophus***. Perhaps, as was done in research for this thesis, their inclusion was done to supplement the medium, to prevent any limitation. Soluble material (recovered from cell separation) is fed back to the culture, supplemented with further carbon and nitrogen. This is good waste management and recycle, provided soluble toxins do not predominate eventually. The main problem of the patents was their repeatability. It can be said, however, that ***Alcaligenes latus*** does look like an interesting proposition, even though it can be difficult to cultivate.

Suzuki, Yamane and Shimizu (1986[41]) described the mass production of **PHB** using a methylotroph. This process eventually led to a fed-batch production, whereby a cell

density of 206g/l was obtained. The **PHB** concentration was 136g/l at this point, some 66% of the dry weight. The organism used was ***Pseudomonas*** **K.** Operating conditions of note were a temperature of 30°C, 2.5±0.5 ppm oxygen concentration and growth on methanol at 0.5±0.2g/l, during fed-batch growth. The residence time of the experiment was notably long, at nearly 200 hours. Additionally, the molecular weight of **PHB** was relatively low, at around $3*10^5$. This is nearly ten times lower than ***Alcaligenes*** or ***Azotobacter*** produced **PHB.** The production also used methanol as a carbon source, which again is a sound economic choice, being relatively cheap and abundant. This experiment was conducted in a 2l vessel, and it is unclear how it would fare in a pilot-plant scale. This level of production, based on earlier calculations, would amount to an annual production of 8.9kg from 2l. To produce about 100 tonnes, this would require a vessel of $25M^3$. Thus, whilst having a cell and **PHB** density higher than anything previously reported, the production was compromised by such a large residence time. It does not represent a suitable production process, therefore. As an academic study, it represents an interesting piece of work.

Doi, Tamaki and Soga (1987[6]) looked at the mechanism of **HV** incorporation into the copolymer of **poly(HB/HV).** By using pentanoic (valeric) acid, they were able to promote a copolymer of up to 90mol% **HV** content. The pathway they

were able to propose has been incorporated into the pathway drawn up in the **Introduction**. By feeding valeric acid, **valeryl CoA, pent-2-enoyl CoA**, and then **L-3-hydroxyvaleryl CoA** are produced sequentially. An epimerase enzyme converts the L into the D form. The **D-3-hydroxyvaleryl CoA** is then built up into the polymer randomly. By varying the relative percentages of valeric acid and glucose, different mol percentages of **HV** were formed, up to 90%. Using 20g/l pentanoic acid, 0.5 and 1g/l glucose, the mol% of **HV** were 73 and 61% respectively. If glucose was omitted, then **HV** went up to 90 mol%. This polymer had a melting point of 108°C, compared to about 175-180°C for pure **PHB**. They characterised the mol% using a 125MHz proton noise decoupled ^{13}C nuclear magnetic resonance spectrum. It is therefore seen that the use of glucose could be a suitable "tuning" agent. This could accurately control the mol% of **HV**, and thus tailor specific polymers. Use of pure **PHB**, could lead to even more specific copolymer blends of specific nature. This work was done at 30°C, and at a volume of only 100ml. A significant scale-up would be necessary to see if the system operated sufficiently.

Byrom (1987[42]), of **ICI Biological Products**, described the synthesis of polymers by microbes. **PHB** was described, of course, along with potential markets for biopolymers in the 1990s. This would account for about 700 tonnes of

adhesive/coating polymers, worth £30Million/year. Fibres would be produced at about 900 tonnes, worth £35Million/year. Polymers used as gums would require a production of 40,000 tonnes, worth £100Million/year. Plastics would require a production of 2000 tonnes, worth £35Million/year. The potential for biopolymers is undoubtedly there. The part that **PHB** plays in this scenario, if any, remains to be seen. An account of production organisms, economics and product recovery is given, with associated problems. It is noted that **PHB** has been produced at the 35 and $200M^3$ vessel scale.

Doi, et al (1987[10]) described the production of copolymers including monomers of **5-hydroxyvalerate (5HV)**, previously undescribed. To create the new monomer, **5-chloropentanoic acid** was used as a co-carbon source, in addition to glucose and pentanoic acid. Cultures were again operated at the 100ml scale. Four feed regimes were performed. Initially, **5-chloropentanoic acid** was added only, at 20g/l. This promoted only 1% polymer storage, with 52% comprised of **5HV**, and 24% as **HB** and **HV** respectively. By using only pentanoic acid, at 20g/l, 46% polymer storage occured, which was 85% **HV** and **HB** as the remainder. When equal quantities (10g/l) of both **5-chloropentanoic acid** and pentanoic acid are used, 8% polymer storage occured. This polymer was 63% **HV**, 11% **5HV** and 26% **HB**. If the **5-chloropentanoic acid** was decreased to 5g/l, and the pentanoic acid increased to 15g/l,

essentially the same polymer is formed, but with 19% storage. No reference was made to the melting points of each copolymer. It is notable that only the **poly(HV/HB)** copolymer accumulates to 46% of the cell dry weight. The cell density only amounted to 7g/l, over 48 hours. This would seem to be a good method to investigate the molecular pathways in ***Alcaligenes eutrophus NCIB 11599***. The majority of this work entailed NMR analysis.

Southgate (1987[43]) (formerly of **ICI Biological Products**) wrote a process description for the production of a copolymer at the $50M^3$ scale. The author of this thesis gained production-scale experience, by being involved with two of such runs. A short description of what occured is given in **Chapter 8.** Included in the report is a list of widely known **PHB** and copolymer applications. This is detailed in the **Introduction.**

2.4 Conditions necessary to grow the relevant bacteria (for **PHB** formation).

Six years after the French study of **PHB** producing ***Bacillus*** ceased, **Schlegel, Gottschalk** and **von Bartha (1961[44])** started to look at ***Alcaligenes eutrophus***. At this time, however, the organism was known as ***Hydrogenomonas eutropha***. The bacteria were cultivated chemoautotrophically, by supplying carbon as CO_2, and including H_2 and O_2. With the inclusion of

0.3g/l NH_4Cl, exponential growth proceeded for about 40 hours, after which protein production remained zero. From 40-110 hours, the cells accumulated 65% of their dry weight as inclusions of a refractile material. Using conventional techniques, such as potassium hypochlorite cell digestion, the material was recoverable. It was soluble in hot chloroform, and insoluble in ethyl-ether or petroleum ether. Thus it was concluded that the refractile material was **PHB**. Microscopic analysis showed that the cells eventually filled with **PHB**. This displaced the nuclear material (chromatin granules) to the cell periphery. The polymer had a melting point of 170°C. The stoichiometry of the reaction was:-

$$25H_2 + 8O_2 + 4CO_2 \rightarrow (C_4H_6O_2) + 22H_2O$$

Organotrophically grown cells also accumulated **PHB**. If **PHB** rich cells were fed NH_4Cl, then respiration was maximal. **PHB** was eventually degraded, under the circumstances, as protein formation restarted. This was one of the first papers whereby non-spore formers were examined, and **PHB's** role was determined and accumulation characterised. In this instance, **PHB** was described as being a storage material, which could provide a carbon source, and a potential for protein synthesis if nitrogen was present. The paper also described the conditions necessary to grow a new genus, which the author of the thesis used in this study.

Schlegel, Lafferty and Krauss (1970[45]) examined deficient mutants, unable to produce **PHB**. This was conducted in order to examine the molecular biology of **PHB** formation in ***Alcaligenes eutrophus*** **H16**. Mutants were induced, and then selected by **Sudan Black** staining. **PHB**-less mutants remained unstained, and were isolated to examine the mechanism of **PHB** formation, compared to the wild-type strain. The paper outlined methods to produce mutants, and recover them from the wild-type strains, in order to perform further experiments.

Repaske and Repaske (1976[46]) provided very detailed information for the exponential growth of ***Alcaligenes eutrophus***. This concerned the growth of the bacteria in 23l vessels, under an autotrophic environment. One way of creating higher cell densities, could be to increase the starting concentrations of minerals. This would then avoid the possibility of rapid nutrient limitation. The problem of doing this is that too high concentrations can be toxic. By supplying controlled additions of required minerals, the cell densities were increased from $6\text{-}8*10^9$ cells/ml to $8*10^{10}$ cells/ml. **PHB** formation occured as a result of magnesium or sulphate deficiencies, as well as other previously known chemicals (O, N and P). A need for three trace elements was determined in autotrophic growth of this bacteria (Ni; which contained Co contamination, Cr and Cu). The first three have been incorporated into

the growth medium for this thesis research (Cu was already present). Whether or not they are required in organotrophically grown cells (or they were also present as contaminants in other minerals) it was felt prudent to include them. Caution was required not to overdo this, as slight increases in concentration actually hindered growth. The use was such that it provided against limitation, and used correctly, growth was not harmed. Experimentally, it seemed unlikely that growth was stimulated, probably though contamination of other minerals meant they were already present. However, inclusion guaranteed they would not run out. The cell densities were measured spectrophotometrically at λ of 660nm. This is convenient, as it allows a direct comparison with this thesis research. The maximum absorbance was about 40, which corresponded to a cell density of $8*10^{10}$ cells/ml. This is equivalent to the best densities reached in this research. A growth rate (μ) of $0.35-0.4h^{-1}$ was achieved, which was slightly less than the present research, reaching $0.5-0.55h^{-1}$, under organotrophic growth. In creating minimum saturating concentrations for autotrophic growth of ***Alcaligenes eutrophus***, the paper was particularly valuable.

Repaske and Mayer (1976[47]) reported the production of dense autotrophic cultures of ***Alcaligenes eutrophus***. They produced a 25g/l cell dry weight culture,

corresponding to an optical density (or absorbance, at 660nm) of 60, and a cell density of $1.2*10^{11}$ cells/ml. This occured over 25 hours incubation, which was run as a batch or fed-batch cultivation. Certain minerals were added throughout (due to the toxicity of too high start concentrations) and a growth rate (μ) of $0.2h^{-1}$ was attained. Exponential growth continued for 19 hours, until pH control led to an excess of NH_4^+ ions, which decreased the growth rate (μ). By automating the control processes, it should be possible to get higher densities. Providing such a high **PHB**-free biomass density, could lead to the production of cells to the range of 50-250g/l, containing substantial **PHB** quantity. This is based on research, in which after limitation, cell weight can increase by two-ten times, whilst **PHB** accumulates. It has already been reported that a cell density of 206g/l has been achieved (containing 136g/l **PHB**, in a fed-batch culture of ***Pseudomonas K***). The culture time was nearly 200 hours, therefore control apparatus and computer linkage will play a significant part in creating dense cultures in shorter times.

Lafferty (1979[48]) produced an American Patent, which produced methods to isolate good **PHB** accumulating strains. This represented work done in conjunction with **Agroferm**, Switzerland. Mutagenic methods were employed, to see whether particularly useful strains could be gathered. Various bacteria were used, namely ***Bacillus***

megaterium, *Pseudomonas* B79 NCIB 9088, *Pseudomonas* B175 NCIB 9089, *Pseudomonas facilis* and *pseudomallii*, *Chromatium violaceum*, *Rhizobium* HCCB 142, *Azotobacter beijerinckii*, *vinelandii*, *agilis* and *chroococcum*, *Alcaligenes eutrophus*, *Flavobacterium aquatile*, *Zoogloea ramigera* and *Mycoplana rubra*. Cultures staining heavily with **Sudan Black**, on agar plates, were used for further investigation.

Friedrich, Bowien and Friedrich (1979[49]), also of Göttingen University, West Germany, experimented on *Alcaligenes eutrophus*. They were interested in studying oxalic and formic acid metabolism, in this representative of the hydrogen bacteria. The growth rates were much lower than previously reported, using fructose and pyruvate. Formate metabolism proceeded with the formation of CO_2, which was then fixed by the **Calvin cycle**. Oxalic acid was used organotrophically. **Friedrich, Friedrich and Bowien** (1980[50]) examined the formation of autotrophic enzymes, during heterotrophic growth. Under sub-optimal conditions, when energy requirements were not fully met, enzymes of the **Calvin cycle** are formed. With pyruvic, succinic and acetic acids, no autotrophic enzymes are formed. Fructose, gluconic or citric acids, used as the carbon source, provided a 20% stimulation of autotrophic enzymes. Growth on formic acid and glycerol led to the formation of autotrophic enzymes, comparable to autotrophic growth levels. *Alcaligenes eutrophus*

is a facultative autotroph, which can fix CO_2 or use organic carbon. This, along with **PHB** formation, is an evolutionary advantage. It is also further evidence that algae and higher organisms were originally derived from prokaryotes.

Siegel and Ollis (1984[27]) looked at the effect of oxygen inhibition on autotrophic continuous culture. Monod equations on dissolved O_2 concentration and H_2 concentration, enable details on O_2 toxicity to be determined. In addition, by severe limitation of O_2, **PHB** production was again produced to over 20%. This strain of ***Alcaligenes eutrophus*** was not as good at producing **PHB**, but reveals further evidence on metabolism and culture techniques.

2.5 Physiological role of PHB formation.

Tsukagoshi and Arima (1967[51]) provided one of the first reviews on the role of **PHB**, physiological function, synthesis and physical properties. In a meeting of the Biochemical Society, **Dawes** (1967[52]) described the production of storage materials, in stationary phase cells. **Dawes and Senior** (1973[2]) published a 131-page review on the role and regulation of energy reserve polymers. This included criteria for the function of storage materials (including details on polyphosphate and glycogen storage). Why do cells accumulate reserve

material? Obviously in times of nutritional stress, the occurrence of a metabolisable storage material is highly advantageous.

The criteria for storage of energy reserve materials were:-

(i) the material must be formed, when the exogenous energy source is in excess of that required by the organism at the time.

(ii) when the exogenous source of energy was depleted, the material must be used to provide energy, for maintenance or growth.

(iii) the energy formed upon degradation, must be in a form utilisable by the organism. This will give the cell a biological advantage, over those that can not store such material.

Different cells accumulate either one or several polymers. The first statement of note was that **PHB**, unlike the other compounds, does not require the utilisation of **ATP**. The work of Lemoigne's group in France was acknowledged, and a description of the occurrence of **PHB** in bacteria was given, including relevant references. Physical properties of **PHB** were also reiterated. **PHB**, it is stated, is an excellent storage material, existing in a highly reduced form. It is an insoluble crystalline polymer, which does not effect the

cells osmotic balance. In addition, the acidic intermediaries are removed from the cell interior. The specific roles of **PHB** were sporulation in ***Bacillus*** species, energy and carbon sources in ***Azotobacter*** species. In aerobic organisms, which frequently find themselves in low O_2 concentration zones, **PHB** is stimulated. This is made more dramatic by the fact that O_2 actually affects the growth of these aerobes detrimentally. Thus **PHB** is a safeguard for the organism, which has to live in a specific ecological niche, such as found in the rhizosphere. This review is very important, in elucidating many facts surrounding **PHB** formation, and even degradation. **Emeruwa and Hawirko (1973[8])** elucidated a role in sporulation of ***Clostridium botulinum***. This provided a comparison to the sporulation of ***Bacillus*** species. The anaerobic ***Clostridium*** genus, considered more primitive, has a similar use for **PHB** in sporulation. The authors characterised **PHB** formation in sporing and spore-free mutants. **PHB** inclusions were even present, to a very limited degree, in the endospores. This itself was used eventually, presumably during germination. Spore-less mutants were unable to degrade **PHB**, suggesting a link between enzymes of polymer and spore formation. The genetic information for both processes is also probably linked, possibly explaining the lack of degradation.

Shively (1974[3]) described **PHB**, in his review of

inclusion bodies in prokaryotes. **PHB** granules are normally 0.1-0.8μm in diameter, and consist of 98% **PHB**, 2% protein and trace amounts of lipid and phosphorus. The protein represents the enzymes responsible for polymerisation and degradation. The lipid represents part of the surrounding membrane, which is about 2-4nm thick. This is a single layer of the cytoplasmic membrane. There is evidence that in ***Bacillus cereus***, the granule has a central core. This shows the various polymerisation stages, and the state of the polymer chains. Molecular structure studies indicate **PHB** is a crystalline right-handed helix. The helical nature of the polymer is seen in the **Introduction** (along with copolymer structures). The helix has a repeat unit every five monomers, which is in agreement with current thinking.

2.6 **PHB** determination in cell material.

The earliest references to **PHB** determination were, not suprisingly, introduced by Lemoigne. In 1926, he developed a gravimetric method, based on **PHB's** solubility in hot chloroform. The polymer was precipitated (in addition to using other solvents to remove contaminants), and pure samples recovered. However, large quantities of cells were obviously required. Williamson and Wilkinson developed a process to recover **PHB** from cells, in 1958. Biomass was dissolved in sodium hypochlorite, and the

turbidity of the lipid granules was measured. Law and Slepecky in 1961, developed a spectrophotometric method. By degrading extracted polymer in concentrated sulphuric acid, the extinction coefficient of crotonic acid allowed an absorbance figure to be calculated. Crotonic acid absorbs light at a wavelength of 235nm. The technique was lengthy, and also required relatively large samples. These tests were again described briefly by **Dawes and Senior (1973[2])**, in their review paper.

Ward and Dawes (1973[53]) developed a disk assay for **PHB**. Instead of using large samples, and centrifuging repeatedly, as occured in the method of Law and Slepecky (1961), glass fibre disks were used. Cell cultures were applied to the disks, which then facilitated easy hypochlorite and sulphuric acid degradation. The crotonic acid produced gave identical results to the 1961 method, indicating the suitability of the process. The major advantages over the previous method, was that smaller samples could be used, containing less **PHB**. Additionally, centrifugation was removed, which both speeded up the process, and allowed greater sampling. The only problem was that of the sensitivity of the spectrophotometer, and interference from several bacterial components. It was a useful (analytical) process development, however. Two years later, **Jüttner, Lafferty and Knackmuss (1975[54])** proposed an **IR spectrophotometric** method for **PHB** determination. **PHB** was dissolved in chloroform (after

solvent extraction from the cells) and **IR spectroscopy** was used to calculate **PHB** quantity. The use of silica-gel thin layer chromatography, allowed contaminatory lipid to be discounted. The results showed that purification had been done to a very high standard. **Braunegg, Sonnleitner and Lafferty (1978[55])**, a group now working in Austria, defined a method for the rapid determination of **PHB**. Using gas chromatography, acidified methyl esters of crotonic acid were injected into the column. Residence times of pure sodium salts of hydroxybutyric acid were identical. Cell samples were used directly, avoiding a separation step, by heating a solution of acidified methanol and chloroform. The technique is described in full in **Chapter 4, Section C.2.4.**

Nickerson (1981[56]) described a **PHB** purification technique, using sodium bromide density gradient centrifugation. This process represented a method to recover **PHB** granules (membrane bound or free) without interference from other cell material or spores. The Law and Slepecky extraction method was used, and the technique, overall, is not all that useful. **Srienc, Arnold and Bailey (1983[57])** used the principle of flow cytometry, to characterise **PHB** inclusion in ***Alcaligenes eutrophus***. Cells having come from the stationary phase have a relatively long lag phase, when reincubated. The scatter density decreased, as **PHB** inclusions were degraded. As cells grew exponentially, became nitrogen

limited and then formed **PHB** again, the scatter intensity increased. The technique provided a way of investigating the heterogenous nature of cultures. It also provided a very useful method for dry weight calibrations. Significantly, the best use of the process probably lies in enrichment culturing. As cells were exponentially growing (**PHB** storing or deficient ones), different forms could be enriched. The scatter measurements would help "weed-out" unwanted forms.

Pedrios-Alio, Mas and Guerro (1985[58]) looked at the effect that **PHB** had on cells buoyant density. Using sucrose as a density gradient material was not suitable for **PHB** accumulating cells. Due to osmotic effects, sucrose graded cells apparently showed **PHB** containing cells to be less dense than **PHB** free cells! Using **Percoll**, exponentially growing cells were seen to be less dense and of less volume. When nitrogen limitation started, the cells increased in both density and volume. Relationships between **PHB** versus cell volume, and **PHB** versus buoyant density were formed. This work was again useful for academic, rather than quantitative work.

Two **ICI** in-house **PHB** determinations have been developed. **Mann** (1985[59]) proposed a mechanism for perchloric acid **PHB** degradation. The monomer is subsequently formed (**D-3-hydroxybutyric acid**), which is converted into acetoacetate. **Nicotinamide adenine**

dinucleotide (NAD, oxidised form of NADH) and the enzyme **D-3-hydroxybutyric acid dehydrogenase** are required to complete the reaction. With the presence of **hydrazine**, **hydrazone** is formed. This removes the acetoacetate, which tends to reform the monomer, under suitable conditions. The reaction for monomer to acetoacetate is thermodynamically favourable with respect to the monomer. The actual measurement is a spectrophotometric assay of **NADH**, and it is necessary to prevent monomer reformation, as **NADH** would be reconverted back to **NAD**. If higher concentrations of **NAD** are present, along with a buffer at pH 9.5, acetoacetate and **NADH** are formed. It is seen that the amount of **NADH** formed is directly related to the initial polymer quantity. **NADH** is measured at 340nm in a spectrophotometer. The adapted version was written by **Johnson (1986[60])**, and is currently used at Billingham. The process is run on an autoanalyser, which can deal with a number of samples at a time. This measures the maximum absorbance over 1 hour. The measurement of cell samples containing copolymers can give problems. If **3-hydroxyvaleric acid (HV)** is present, a reduction in absorbance arises, compared to a pure **PHB** sample, of the same weight. This is because the enzyme is highly specific to **PHB**. If **HV** is present, the enzyme works less efficiently, and less **NAD** is reduced to **NADH**. As part of the **"CASE"** award industrial session at **ICI**, the author of this thesis described a solution by use of a correction pattern. This is described fully in **Chapter 8**. This

process is probably the most efficient and accurate way of determining **PHB** quantitatively.

Doi, et al (1986[61]) describe the results of nuclear magnetic resonance (**NMR**) studies on **Poly(HB/HV)** copolymers. **NMR** studies of copolymer can quantitatively determine the mol% of **HV** and **HB**. Additionally, reverse phase high performance liquid chromatography (**HPLC**) can also be used to gauge mol% **HV** and **HB**. This was described in **Chapter 4, Section D**.

2.7 **PHB** recovery; extraction processes.

Lemoigne (1926) noted that **PHB** could be extracted by hot chloroform. Williams and Wilkinson (1958) described the recovery of **PHB** by hypochlorite extraction. Both these works were done to determine the amount of **PHB** in cells. The first industrial recovery of **PHB** was conducted by **Lafferty and Heinzle (1979[62])**. Lafferty had previously worked with Schlegel, who published work on **PHB** production in 1961. Chloroform was again chosen to recover **PHB**, but has the inherent disadvantage of removing contaminatory lipids. This provided the impetus to create a more satisfactory process. In collaboration with **Agroferm**, Switzerland, Lafferty and Heinzle used various cyclic carbonic esters to extract **PHB**. The various test solvents solubilised **PHB** to differing extents. In some solutions, where **PHB** was only just

soluble, the solutions were very viscous. Others, like ethylene and propylene carbonate, dissolved **PHB** at 120 to 150°C. The resultant solutions were filterable from cell material. Using these two solvents, **PHB** fibres were spun at 8M/minute, using a precipitating water bath of 1:1 water:solvent. Ethylene carbonate was less suitable, as it significantly degraded the molecular weight (down to 120,000 daltons). 1,2-propylene carbonate extracted polymers of $5-9.5\times10^5$ daltons. The percentage recoveries were also 89-96% respectively. These figures were after three hours and 52 minutes incubation respectively.

ICI entered the fray, with **Holmes and Wright (1981[63])**. He proposed the use of chlorinated hydrocarbon solvents, and also examined propylene carbonate. By spray-drying cells or centrifugation, solvent extraction was performed, with or without a cell shearing step. By concentrating cell material, a solvent "syrup" was formed, containing **PHB**. This was then left to stand, and added to a water/ methanol mixture. This mixture allowed **PHB** to flocculate out of solution, as white, fluffy, fibrous material, of very low density. **Holmes, et al (1979[64])** looked at the introduction of a primary solvent extraction. This would be used to extract non-**PHB** lipid. The primary solvent step proposed the use of a 5-minute acetone or methanol reflux step. The secondary solvent extraction step used chloroform or dichloroethane. **Walker, Whitton and Alderson (1980[65])** further adapted the

principle, using a steam injection/flocculation step. This was done on ex-fermenter biomass, prior to solvent extraction. This allowed a very efficient extraction, to about 95-98% of the **PHB** in cells. The problem with this system was the recycle of organic solvents. **Holmes and Jones (1980[66])** proposed a further adaptation, by disintegrating cells using pressure and heat and recovering **PHB** granules. These were purified further using solvents to dissolve the lipid coats only. Use of electric fields and pH changes, allowed good separation of **PHB** from cell debris.

Lim and Holmes (1984[67]) examined the extraction of **PHB** using a new technique of enzyme digestion. The ex-fermenter cell solution was flocculated (by steam injection) and centrifuged. The solids were resuspended and treated with various enzyme preparations. These severely weakened the cells. They were centrifuged and resuspended again. The "leaky" cells were finally disintegrated using a detergent, which solubilised all non-**PHB** material. This was centrifuged, resuspended and treated by peroxide, to bleach the polymer. The **PHB** was filtered and dried. The final procedure was formalised by **Southgate (1987[68])**. It has been patented **(1987[69])**, in an unknown patent application. The author of this thesis examined the efficiency of this procedure, whilst at Billingham. The results are described in **Chapter 7**.

2.8 Biodegradable polymers.

The realism that **PHB** had the properties of a thermoplastic polyester, made the importance of biological creation paramount. It was logical to surmise that if bacteria could produce **PHB**, they would be vastly more able to degrade and utilise it. Elucidation of the metabolic pathways confirmed the formation of **PHB** to be cyclical. Thus enzymes for degradation were naturally present. In fact, when Peter Senior buried a poor solvent-cast film of **PHB** (in a rose bed at Hull University), it soon became apparent how biodegradable it was. To create a commercial thermoplastic polyester which was biodegradable, has an obvious potential and importance. Previous studies have shown that a variety of bacteria (and especially fungi), are able to degrade **PHB**.

Subcutaneous implantation into rabbits, led to complete absorption within three months. The later study of 1966, showed the potential for **PHB** in medicine. The single most important fact of this, was that no immune response was elucidated to **PHB**. The **hydroxybutyric acid dehydrogenase** enzyme is present in mammals naturally. The **ICI** document which **Holmes, et al (1983[70])** formulated, examined only soil degradation of films. Several films were produced; melt cast polymer granules, solvent cast films and a **PHB** paper. Two types of soil were used, one of an average composition (both in minerals and

microbes), and another extreme one (which had greater microbial action). After incubation in wet soil, at 25°C, scanning electron microscopy showed surface degradation (by fungal mycelia). The paper films were attacked fastest. Degradation was quickest in wet soil, with higher microbial action. After one year, all of the samples (6" square) were too degraded to recover. Little evidence of bacterial attack was detected, however. **Robson and Holmes (1984[71])** further demonstrated film degradation. Various copolymer compositions did not affect degradation rates. Molecular weight influences the rate; if a polymer with a ten times drop in molecular weight was examined, a 50% increase in degradation was seen. If plasticisers are used in formulating moulded polymer, then water soluble ones enhanced attack. This research was of a preliminary nature, and a small number of samples were used. These are then statistically less significant. Another problem is the duration of the experiments, sometimes lasting 8-12 months, which does not help speedy development.

ICI Pharmaceuticals (in conjunction with **Liverpool Polytechnic**) investigated the production of a polymer drug carrier, which was biodegradable. This is marketed as **ZOLADEX**, which is used to treat prostate cancer (**Rostron (1988[72])** and **Rak, et al (1985[73])**). The polymer carrier is made up of monomers of lactic or glycollic acids. This polymer (or copolymer) has also been tested

satisfactorily as sutures and prosthetic implants. Again, no toxic or immune effects were observed. The cost of this product is very high, the synthetic manufacture proceeds in sterile conditions, and only entails the production of kilograms/year. **Lafferty, et al**, have also examined **PHB** as a drug delivery matrix. It is not known whether comparable tests of **ICI** "BIOPOL" and "ZOLADEX" have been performed. Such a comparison, if not already available, is needed.

Tunc (1986[74]) investigated the use of similar (poly lactic and glycollic acids) polymers, and characterised surgical use. No cytotoxic effects were again observed in either mice or rabbits, when small samples were implanted. High density polyethylene control samples remained unchanged after two and a half years. The test samples were degraded by 70% in such time. To repair broken bones in dogs, Tunc examined the strength of the repaired join, some two years post-surgery. Polylactic acid repair pins allowed 100% of bone strength to be retained, compared to unbroken bone strength. Steel pins and plates effected only a 23% strength recovery. As the polymer degraded, the bone fibres grew through the polymer, and rejoined fully. Steel, on the other hand, remained in place, needing to be surgically removed. Consequently the bones were less well recovered. The technique, however, required the artificial, clinical breakage of healthy bones. These live dogs were then

slaughtered for study. If the process had been followed in non-clinically broken bones, then the study would have been more acceptable. However, without it, less information would be gained. This might be a small price to pay, as comparative stress testing could be done in live animals.

2.9 Moulding, quality control/quality assurance techniques.

The earliest patent pertinent to the industrial use of moulded **PHB** (containing cells), was filed by **Baptist and Werber (1960**[11]). These workers were employed by **Grace and Co.**, New York, USA. **PHB** was known to be formed by **Pseudomonads**, ***Spirillaceae***, ***Rhizobiaceae***, ***Bacillaceae***, and ***Azotobacteriaceae*** families. In this instance, a ***Rhizobium*** species was used, to produce **PHB** to about 40-95% of cell dry weight. Pyridine was used to recover **PHB**, reasonably successfully. Compression machines, operated at high pressure and temperature (10,000 psi and 190^{o}C), produced light brown sheets of plastic. Sometimes extracted **PHB** was mixed with cells, prior to moulding plastic. If formaldehyde was used, then water resistance of the moulded sheets was improved. This was analogous to producing industrial plastic using protein (casein). The **PHB** which was formed, was of "high molecular weight", although what this actually represented, is unknown. **PHB**, it was stated,

could be used to produce a variety of plastic articles, but the biodegradable aspect was not mentioned. At this time, of course, the study of molecular physiology of **PHB** production, was generally in its infancy.

Nickerson (1982[56]) demonstrated the use of NaBr density gradient centrifugation as a suitable separation process. Different molecular weights of **PHB**, cell debris and spores could be isolated. **Holmes** (1983[75]) described mechanisms to study melt-flow characteristics of **PHB**. This enabled the extracted polymer to be processed. **PHB** powder was passed through a screw extruder, which heated and compacted the polymer. The extrudate was a brittle lace, which could be run through a water trough, to allow crystallisation. The crystallised lace was then pelletted, bagged and stored. **Henman and Holmes** (1982[76]) examined the use of chemical additives, which could stabilise, nucleate and modify polymer. If chlorinated polyethylene was added to **PHB** (in 10 and 40% ratios), then the resultant plastics were weaker but more heat resistant, or vice versa. Specific applications of polymers could then be tailored precisely. **Barham and Wills** (1983[77]) looked at the effect of nucleation behaviour in **PHB**. Two effects of nucleation were described, by kinetic study. The effects of nucleation are necessary to get a proper crystalline polymer, and various nucleating agents were examined. The precise nature of this is outside the scope of this thesis, and

is related to polymer chemistry and physics. Further discussion will not be given, therefore. **Barham, et al (1983[78])** further developed a series of studies on **PHB** properties. It has importance to the study of polymer characteristics, on a more widespread scale. Nucleation and crystallisation studies of **PHB**, as it is so pure, could provide a model for other polymers. **Holmes (1983[75])** again put forward results of studies on melt stability, nucleation and crystallisation, extrusion and injection moulding. Additionally, a study of fibre spinning was reported, for the "BIOPOL" range. This has presented problems, particularly for **PHB**, due to its physicochemical properties. Some success has been gained, but copolymer structures could help matters. The structures of **PHB** and copolymers, shown in the **Introduction**, give an idea into the likely spinning problems. When **PHB** was spun, then the thread line frequently broke. As the polymer recrystallises and relaxes on cooling, the structure tends to favour an aggregate, rather than a chain. The use of the **Chem-X** program, operated at the **Polytechnic of Wales**, might provide information on likely spinnable polymers. This program was used to generate the models depicted in the **Introduction**. It can be used to optimise structures, rotation of atoms creates the most thermodynamically stable structure, eventually. Minimum energy profiles could be created, to show suitable structures. If microbial production of such molecules was possible, then

further classes of applications would appear. This would involve a great deal of work, if it was at all possible. **Bloembergen, et al (1986[79])** examined the composition of copolymers of **poly(HB/HV)**. The effect of composition on crystallinity was also followed. Nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FTIR) were used to analyse the composition.

Other quality control/assurance techniques involve DSC, GPC, MFI and GC (differential scanning calorimetry, gel-permeation chromatography, melt flow index and gas chromatography, respectively). These are all described in **Chapter 4, Section D (Analytical techniques to assess polymer quality).**

2.10 Application of **PHB** and **PHB** structure.

Lundgren, Pfister and Merrick (1964[80]) described detailed microscopic analysis of **PHB** granules. Their electron micrograms show polymer enclosed in a "delicate, skin-like structure". Typically, **PHB** granules are spherical, but were occasionally rod-like. The diameter (ϕ) varied between 0.2-1.1μm, isolated from ***Bacillus cereus***. Crystals of **PHB**, formed by dissolving in chloroform, and treated with ethanol, are lathe shaped. This is apparently similar to extruded crystalline polymer, free from the lipid membrane.

Korsatko, et al (1983[81] and 1983[82]), describe the use of **PHB** as parenteral matrix tablets, for controlled drug delivery. Using such matrices, the drug **7-hydroxyethyltheophylline** was released, in a controlled linear manner, over 50 days, in vitro. Subsequent biodegradation studies of **PHB**-matrix drug-carrying tablets, followed the release and degradation over 20 weeks. This was conducted in mice, by subcutaneous implantation into skin-folds of the neck. The in-vivo (in mice) degradation rate was about two-three times slower. This figure, however, depended on the size and surface area of the implants, as would be expected.

Senior (1984[12]) described how "I fondly believed I had got the world's first biodegradable, melt processable, oil-independent thermoplastic polymer in my hands". This had come about due to the burial of a poor film of **PHB** in soil. **PHB** had a useful advantage over synthetic polymers, in that it was totally biodegradable. The cost of production tended to overshadow this fact, though. The predicted market was that of the "low volume, high cost" sector. This would include the controlled release of drugs and agrochemicals. The fabrication of articles which pose a severe environmental impact, could be rivalled, if costs were reduced sufficiently. The biocompatability of the medical use of **PHB**, in sutures, pins, etc, was also mentioned. Because of several other properties, **PHB** could be a useful polymer film. The gas

barrier properties, which could be used to prevent oxidation or CO_2 damage, would allow food and beverage applications. The piezoelectric effect of compressing **PHB**, could be utilisable in various electrical applications. The initial degradation of **PHB**, would provide a suitable mechanism for recycling the polymer. This would be analogous to recycling glass bottles, for example.

Lafferty, et al (1984[83]), described the production of **PHB**, and it's applications. Unlike **ICI**, they used ***Alcaligenes latus*** to make only pure **PHB**. The use of **PHB** as a matrix material for drug delivery, was probably pioneered by the Austrian group. The monomer of **PHB** could also be used in synthetically forming speciality chemicals.

Holmes (1985[13]) described a review of **PHB** applications. Another application could be the replacement of glucose drips in medicine, by feeding **HB** monomers orally. **"Chemistry In Britain" (1987[84])** mentioned a tongue-in-cheek use for **PHB**: the use of plastic shrouds to bury bodies in! **ICI Biological Products** own prospectus also mentions some of the "BIOPOL" (the trade name for **poly(HB/HV)** copolymers) uses. Two articles in the ***Times*** also mention **PHB** applications, as drug and agrochemical carriers **(3/11/87 and 13/11/87)**.

Thus the story of **PHB** (and copolymers) has been described, in some length. Various categories have been identified (production/moulding etc.), which provide a reasonably in-depth review. It is anticipated that industrial production will eventually start, either in **Austria, Japan, America or the UK**. The potential development represents as exciting a discovery as the original plastics and their subsequent development. The eventual outcome is awaited with great interest, by industrialists and environmentalists alike.

Chapter 3 - Scope of the work.

The following lists describe work done to date, progress made and possibilities for the future.

3.1 Literature survey (updated regularly).

3.2 Batch culture studies:-

(a) in shake-flasks.

(b) in a small reactor vessel (1l).

To: (a) obtain a maximum growth rate (μ_{max}), using several previously described media (the object of the research was not to get the perfect medium) and relevant statistical analyses.

(b) examine primary scale-up, from a 100ml working-volume flask, to a 850ml working volume reactor.

3.3 Fed-batch studies:-

Subject to 3.2, when a suitable medium (or media) was identified, fed-batch studies commenced, using 1 and 2l reactor vessels. In order to do this, routine analytical techniques were adapted, in order to measure primary parameters. Carbon substrate was fed only, to constitute fed-batch, after a period of exponential (batch) growth.

Fed-batch was a term introduced in 1973, to refer to a batch culture which is fed continuously with nutrient medium. In this respect, it is analogous to continuous culture without the outflow. Normally, the amount of culture would be small, or the addition would be a little concentrated medium, so as not to overflow the vessel. Initially, the first fed-batch experiments described in this thesis, used several intermittent additions of strong glucose solution. Later experiments utilised a flow of concentrated glucose, which was fed at the end of the exponential stage, and lasted for at least 24 hours. In this way, the experiments are considered "fed-batch". This was used to promote **PHB** synthesis. Full data recording and presentation techniques were adapted or devised.

3.4 Copolymer production;-

Complementing 3.3; fed-batch growth on mixed carbon feed, during the fed-batch phase, to produce **poly(HV/HB)** copolymers, using two different strains from that used previously. Additionally, the effect of temperature on growth and polymer synthesis was observed briefly.

3.5 Comparative use of a different species:-

The growth rates of ***Alcaligenes eutrophus*** **H/16 S301/C5,** ***Alcaligenes eutrophus*** **TRON** and ***Alcaligenes***

latus were compared briefly. This was done to examine the potential of *A. latus* as a rival production system.

3.6 Continuous culture (chemostat) experimentation.

Examination of continuous culture was done to assess both the biomass and polymer production efficiencies.

3.7 Two-stage work.

Two fed-batch reactors were operated together, in order to examine the possibility of creating semi-continuous polymer production. The first stage generated biomass, and the vessel was almost emptied and refilled, continually. The second stage was used to produce polymer. The first vessel was five or ten times smaller than the second.

From these results, one of the following strategies would be employed to produce PHB, continuously or semi-continuously:

(i) Batch grown biomass, supplying fed-batch polymer synthesis. Cells were grown in a 2l batch reactor, then used to "seed" a 16l fed-batch reactor. The batch reactor would be grown, almost emptied, refilled and regrown

continually. The fed-batch (**PHB** production) vessel would be seeded, and exponential growth would take place. The cells would then store **PHB**. The fed-batch vessel would be drained completely and restarted.

(ii) Two-stage continuous culture. A first stage 1l chemostat vessel would supply a 10l production vessel. As the flow rates would be the same for both, the initial vessel would have a higher dilution rate. This would favour **PHB**-free biomass, which would supply the second chemostat. The production vessel's dilution rate would be appreciably lower, and thus the residence-time would be increased. This would be conducive to polymer storage.

(iii) Two-stage continuous culture -> fed-batch.

The first vessel, once more 10% of the capacity of the production vessel, would produce a continuous stream of **PHB**-free biomass. This would be fed into the production vessel, as a seed, and cells allowed to grow exponentially. Before the cells reached the stationary phase, carbon would be fed. Once suitable time had elapsed for adequate **PHB** synthesis, it would be drained and restarted. Ideally, one first stage vessel would drive a series of second stage ones, so that at any one instance, after a reasonable start-up, there would be product recovery occurring continuously. A process model was devised to describe the optimal system, with reactor sizes, productivity and costs calculated.

3.8 Downstream processing.

An examination of current technology, in this respect, was carried out. This was done partly at **ICI**, during the industrial session of the CASE award.

3.9 Polymer characterisation.

A brief examination of **PHB** and copolymers was carried out at **ICI**. This was done using routine quality control and assurance techniqes.

3.10 Optimal dissolved oxygen tension (DOT) experiments.

One of the supervisors now works at **Bradford University**, and has conducted a parallel research project. One of the aspects of this is the effect of DOT on growth rate. The provisional result of this, in terms of % dissolved oxygen, was put into practice in the latter work conducted in this research.

3.11 Production-scale experiments, using 0.5, 5 and $50M^3$ reactors, to produce polymer.

Two production-scale **PHB**-formation experiments were attended, courtesy of **ICI**. This provided a unique chance to gain large-scale production experience. It helped provide an insight into the inherent advantages

and drawbacks, associated with such large-scale industrial production systems.

Work considered but not completed, due to time constraints, in terms of the original and subsequent project definitions:-

(a) effect of different carbon sources on growth and polymer production.

(b) effect of pH on growth and polymer production.

(c) synthesis of other monomers by different species, and associated screening techniques.

(d) screening for copolymer production.

(e) induction of copolymers by substrate feed.

(f) anaerobes as a source of **PHB** (or like polymers).

(g) use of an air lift reactor, for comparative examination, versus continuous or batch vessels.

(h) computer simulation of batch and continuous **PHB** production, using programs such as **ISIM** and **ACSL**, as a predictive tool.

(a), (b) and (e) were partially observed, whilst (c), (d), (f) and (g) were not considered. (h) was considered, but due to lack of time, it was not sufficiently examined. To examine a variety of organisms, producing a variety of polymers, would really have been either too superficial, or too ambitious.

Further work.

Future research into **PHB** and copolymer production, should encompass these themes as part of the overall research strategy.

(i) computer modelling of **PHB** production, using kinetic models. This would be used to predict interesting research areas.

(ii) comparative use of ***Alcaligenes latus***, to examine it's **PHB** formation capacity. The previous work using this bacteria has not been verifiable. It is difficult to cultivate, and has only been grown on salt-free nutrient agar. It is claimed to be able to store **PHB** to extremely high levels, and in a much shorter time.

(iii) (a)-(g) as described previously. Investigation of (a) would allow greater economic flexibility and protection of an industrial process. The effect of pH should be characterised, to optimise current processes. Screening for novel monomers, copolymers and induction procedures would be necessary, and would demonstrate a responsible research commitment. Examination of anaerobic **PHB** storing bacteria, would also improve the wider understanding of polymer storage. The use of an air-lift fermenter should be considered, from a theoretical point

of view. If subsequently it appeared feasible or advantageous, testing should occur.

(iv) A test of the process model devised in **Chapter 10, Section 2**, would be necessary.

(v) Market research and product development should be undertaken, ideally from an industrial point of view.

Chapter 4 - Materials and Methods

Section 4.A.1

Bacterial cultures used for experimentation.

1. ***Alcaligenes eutrophus*** **H/16 S301/C5**, a glucose utilising strain, was kindly supplied by **ICI Biological Products**, Billingham, Cleveland. This strain was used for the majority of the experiments.

2. ***Alcaligenes latus*** **NCIB 12189 (DSM 1123, ATCC 29714)**, a sucrose utilising organism, obtained from the Torry Research Station, Aberdeen (National Collection of Industrial Bacteria). This was used very briefly as a comparison to 1.

3. ***Alcaligenes eutrophus*** **H/16 S301/TRON**, a variant of C5, This strain is able to tolerate high propionic acid feed concentrations, which are needed to promote copolymers containing **hydroxyvaleric acid (HV)**. **HV** is a methylated form of **HB**, the native monomer. The structure of these molecules and formation is described in the **Introduction, Chapter 1.**

This bacteria was also supplied by **ICI**, is not obtainable elsewhere, and requires their permission to use. It was developed from 1. above. It was used late in the research program as a further comparison to number 1.

Section 4.B.1

Culture conditions used to grow the bacteria

Media constituents used to cultivate all the bacteria used are found in **Table 3**, on the next page.

Alcaligenes latus was experimented with twice, but it failed to grow. Recently it has been stated that this was more difficult to cultivate than ***Alcaligenes eutrophus*** (**Hbarak, 1988**[85]). Due to limited research time, this was not pursued. However, it can be said that an attempt to grow it followed the same system as that used to cultivate the other two bacteria. Axenic cultures were maintained throughout and the organism was routinely sub-cultured on "Difco" salt-free nutrient agar.

Section B.1.1

Both the ***Alcaligenes eutrophus*** strains were grown in 100ml of a suitable medium, in 250ml Ehrlenmeyer conical flasks. These were incubated at 34°C (or 30°C in the case of ***Alcaligenes latus***), in a Gallenkamp rotary orbital shaker, set to 250rpm. The starting pH was set to 6.8, but was initially not controlled (due to the short duration of these experiments, 7-10 hours, and practical difficulties). The specific examples where pH control took place are outlined in **Chapter 5, Section A.3.2.** Cotton wool bungs allow gaseous exchange, and with

Table 3.

Composition in g/l deionised water	Medium number 1	2	3	4	5	10	11	Alcal latus	Bradford Cont cult
$(NH_4)_2SO_4$	8.65	2.0	4.0	12.0	3.0	2.0	3.0	-	7.1
H_3PO_4 (1.1M), ml	18.0	12.0	12.0	2.4	12.0	3.0	1.0	-	12.0
$MgSO_4 . 7H_2O$	0.6	0.8	0.8	1.2	0.8	0.325	0.4	-	0.8
K_2SO_4	1.5	0.45	0.45	1.5	0.45	0.45	0.4	-	As OH^-
$FeSO_4 . 7H_2O$ (*mg)	1.0	7.5 *	15.0 *	0.1	15.0 *	25.0 *	25.0 *	-	4.17 *
Na_2SO_4 (*mg)	-	-	-	-	-	25.0 *	25.0 *	-	-
Glucose	15.0	15.0	15.0	15.0	20.0	20.0	20.0	-	26.3
Sucrose	-	-	-	-	-	-	-	25.0	-
EDTA	-	-	-	-	-	-	-	-	4.17
$Na_2HPO_4 . 2H_2O$	-	-	-	-	-	-	-	4.5	-
KH_2PO_4	-	-	-	-	-	-	-	1.5	-
Fe(iii)NH_4-Citrate	-	-	-	-	-	-	-	0.5	-
$CaCl_2 . 2H_2O$	-	-	-	-	-	-	-	20mg	-
Trace soln. ml	1 A	0.5 A	1 B	1 C	1 B 1 D	0.5 E 1 D	1 F 1 D	2 G	1 D 0.75 B

Trace soln. g/100ml D.I. H_2O	A	B	C	D	E	F	(mg/l D.I.) G
$CuSO_4 . 5H_2O$	0.0946	0.048	0.15	-	0.3	0.0786	10mg
$MnSO_4 . 7H_2O$	0.467	0.24	0.6	-	1.25	0.4055	-
$ZnSO_4 . 7H_2O$	0.464	0.24	0.6	-	2.5	0.4398	100mg
$CaCl_2$ (*.$2H_2O$)	9.5904	4.704	12.0	-	*5.545	*5.545	-
H_2SO_4, ml, conc.	-	-	-	-	0.1	0.1	-
$CrCl_3 . 6H_2O$	-	-	-	0.030	-	-	-
$CoCl_2 . 6H_2O$	-	-	-	0.0005	-	-	200mg
$NiCl_2 . 6H_2O$	-	-	-	0.015	-	-	20mg
H_3CO_3	-	-	-	-	-	-	300mg
$NaMoO_4 . 2H_2O$	-	-	-	-	-	-	30mg
$MnCl_2 . 2H_2O$	-	-	-	-	-	-	30mg

Key to media used to grow the 3 bacteria.
pH - *A. eutrophus* - Media 1-5, 1 & 2l reactors, KOH: 0.2-0.5M, HCl: 0.2M. 16l reactors, KOH: 3M, HCl: 2M. Media 10+11, 50% NH_4OH solution. Continuous culture medium, (as used in Bradford, Polytechnic experiments with medium 5) - KOH: 0.5M, HCl: 0.5M. *A. latus* - NaOH: 10% (aq.) solution (pH 7.0 & 30°C).
Antifoam - Initially "food-grade" silicone, later poly-propylene glycol.

the shaker action, adequate aeration levels are achieved. The 250ml flasks were run as batch experiments, without any addition other than the inoculum. The inoculum was a small volume of an overnight culture of identical medium, again in a 250ml flask. This had been prepared by inoculating sterile medium, with a loopful of bacteria from a 1-day old nutrient agar plate. Cultures of all the strains were kept on nutrient agar, and subcultured weekly. To prepare for batch, fed-batch and continuous culture experiments in the 1, 2 and 16l LH reactors, the following system was employed:

1) The plates subcultured weekly were used to prepare an overnight culture of freshly plated bacteria.

2) This plate was used to "seed" or inoculate a 250ml conical flask. The "seed-flask" contained the same medium which was used in the experiment (100ml), and was incubated overnight.

3) The seed-flask was used to inoculate the 1 and 2l reactors (50-75ml for 1l, and 100ml for the 2l). When the 16l reactor was used, then the 2l reactor was inoculated and incubated overnight. The next morning the contents of the 2l reactor (1-1.5l) were transferred to the 16l vessel.

Section B.1.2

Batch cultures run in 1l LH reactors were again of 7-10 hours duration, and were done to check the scale-up from 250ml flasks. In the 7-10 hour experiments with reactors and flasks, the exponential phase of growth was measured until cessation. Absorbance and total cell counts were monitored, as described in **Sections C.1.1.1 and C.1.1.2.**

Section B.1.3

For fed-batch experiments (with ***Alcaligenes eutrophus***), 1 and 2l reactors were predominantly used. Initially this was with 1l vessels only. These would be sterilised with 750-850ml of medium. The duration of these experiments was around 50 hours. After such a lengthy duration, with repeated sampling, volume levels decreased appreciably. Caution was applied so that the level of liquid did not get too low. A minimum level for the heating element, pH and O_2 probes to remain submerged was required. In this case though, the heater level was critical. Failure to maintain this could burn the element out. pH was maintained by automatic addition of KOH/HCl (usually in 0.2-0.5 and 0.2M strengths, respectively). The vessel was sparged with air at a rate of 1.5 volumes of air/volume of culture/minute (1.5 vvm air). This aeration rate was later changed to 0.3 vvm air after the

"CASE" award industrial session with **ICI** (0.3 vvm air was sufficient to keep the culture aerated). Agitation in these 500 series reactors was provided by the magnetic stirrer option. This entailed a lower "bomb" impeller, and a 6 vaned paddle situated 2cm below the surface. The stirrer speed was set to 450 rpm, (**Sonnleitner, et al, 1979**[23] and Heinzle, et al, 1980[24]) and the vessel is fitted with internal baffles. A photograph of the 500 and 2000 series LH reactors is found in the **Appendix Section.**

50-hour experiments were carried out, with examination of both growth and polymer storage. The polymer is stored due to nitrogen limitation in this case. Various parameters were checked, the full list was eventually collated from several experiments. Growth was measured (by means of turbidity/ optical density/ absorbance, and total cell count), as was nitrogen, glucose, polymer, dry weight and protein concentration. Glucose levels required monitoring, so that the level never dropped to zero. This provided the carbon necessary for polymer storage. Too much glucose was also avoided, as this was inhibitory. The fed-batch stage occured after the exponential growth had terminated. Initially cells would grow on the medium provided, by batch growth. When growth stopped, and polymer storage began in response to limiting nitrogen, concentrated glucose was fed. For the 16l vessel, 500ml of 300g/l glucose (sterile) was used. This was fed at 20ml/hour, over 24

hours. With 1 and 2l vessels, 5 or 7.5g glucose in 20ml of deionised water (sterile) was injected manually, usually around 24 and 36 hours.

Section B.1.4

For continuous-culture, the reactors reverted to their designed use, as chemostats (there was equal flow of liquid medium into and out of the reactor). These reactors have an overflow weir for medium outflow. In practice, however, it was found more suitable to use a dip-tube and seal-off the exit gas line. Pressure would force liquid out of the reactor, if the level rose above the bottom of the dip-tube. Initially, two experiments with continuous-culture were conducted at the **Schools of Chemical Engineering, University of Bradford.** Experiments were run for several days at a time, with varying dilution rates. The optical density (absorbance) was measured continuously, using an LKB Spectrophotometer linked to a chart-recorder. As the cells grew, the density of the culture was recorded in a flow-through cell. Higher densities were recorded (than using a Spectronic 20 at the **Polytechnic**) before the culture became too dense to measure undiluted. This gave an indication of the growth rate, using a medium as specified in **European Patent 046,344 (Hughes and Richardson, 1982[35]).** Variable flow rates were provided by a Watson and Marlow variable speed peristaltic pump. In

this instance, the stirrer speed was controlled to maintain the dissolved oxygen tension (DOT) at 80% (400-1500rpm range). In addition to using the medium and DOT as specified in the above patent reference, EDTA was used. This was to prevent the medium from precipitating (Fe) during sterilisation. The nitrogen source was changed from 5% NH_4OH to $(NH_4)_2SO_4$, but the concentration of N_2 remained constant. The composition of this medium is found in **Table 3.**

The later experiments conducted at the **Polytechnic of Wales**, were done using medium 5 in **Table 3.** The feed supply bottle of sterile medium was stirred continuously, but was not heated. In these instances, the stirrer speed was again controlled by setting the DOT to 60%. This new optimal level was determined at **Bradford University (Montgomery (formerly Underhill) and Bitar, 1987[86])**. Temperature and pH were as previously described. Antifoam addition in all LH reactor experiments was done manually, or by timed dosage on the 2000 series. This was necessary as the use of a foam sensor was unreliable (on one occasion 100ml of antifoam was added overnight!). To collect samples overnight, a fraction collector was used. The outflow tube was fed into the collector nozzle, which supplied a carousel of test-tubes. These tubes were filled in 10-15 minutes, and contained one drop of concentrated formaldehyde (to stop further growth and cell lysis). The test-tube carousel had a plastic shield

over the tubes, which prevented formaldehyde evaporation. Thus, there was the potential to extract twice as much information. The collection of overnight samples halved the experimental time, and information was also gained on recovery time when flow-rates were altered. All of the experiments were used to gauge which system was most efficient for the (semi-) continuous production of **PHB**. The assessment of this was done by means of an industrial model, found in **Chapter 10, Section 2.**

Section C.1 - Evaluation of growth

To evaluate **Growth**, a definition is needed, with a description of how it applies to bacteria, as opposed to higher organisms. **Growth** can be summarised as "1... development or production (of living things), coming naturally into existence, to arise. 2... increase in size, height, quantity, degree, power, etc. 3... the orderly increase of all chemical components in any biological system." "Growth" in higher organisms can be considered as physical (such as weight or height), or social (human children "growing-up" into mature adults, for example). Medically, growth could be thought of as development. In agriculture, crop production looks at the growth of a field of wheat, for example. Mathematically, growth is considered by degrees, powers and quantities. The study of population would consider the whole growth as a quantity. This last one is usually chosen to study prokaryotic growth.

Studying the growth of bacteria (or other prokaryotic cells), is done by looking at the average from population increases. Typically in microbiology, this is referred to as studying the **specific growth rate**, and **doubling time**. If a single bacterial cell is considered, the problems of using conventional analysis become all too apparent. There is the problem of identification, cells tend to look very similar, as opposed to higher organisms. The

size of a cell will only increase significantly during cell division or sporulation (which only occurs in specific genera of bacteria). Changes in size during cell division are of more academic interest, therefore size is not an adequate parameter to measure. Weight changes can be used to determine growth, providing the component measured remains constant in the cell. This is hampered by the fact that if only one component is increased, a true rate cannot be attained. Such an example occurs during the process of storage. For this research the most obvious one is **PHB**, but glycogen or RNA could suffice. Where the storage of these components is not growth associated, such as **PHB**, then an increase in weight is not indicative of the growth rate. **PHB** is formed in growing cells, but to comparatively smaller levels. Dry cell weight readings can therefore be used only in conjunction with other readings.

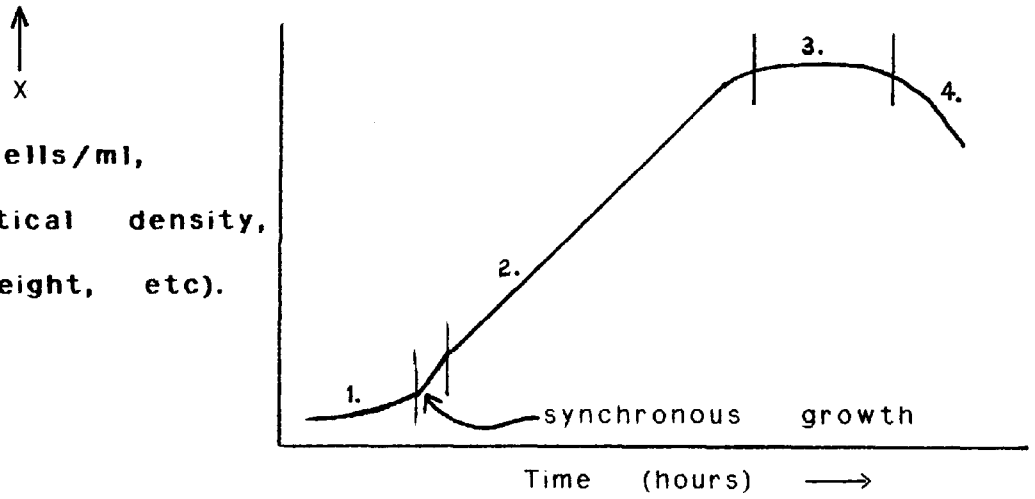
Section C.1.1

Bacterial growth:- Formation of the growth curve, and calculation of growth rate and doubling time.

Considering a bacterial population, under ideal conditions, growth proceeds with four distinct phases:-

1) **Lag phase.**
2) **Exponential or logarithmic growth phase.**
3) **Stationary phase.**
4) **Death phase.**

These are seen if the density of cells in the culture is measured, either in terms of cells/ml or turbidity. In fact, the measurement can include other parameters, the result is the same. Plotting the number of cells/ml versus time will give rise quickly to an exponential plot. This is visualised as 1 cell becomes 2, 2->4, 4->8, 8->16, etc. Initially the increase is small, but before long it becomes very great, rising exponentially. Due to this plot, it is usual to use the **natural log (ln)** of cells/ml. Now the exponential curve becomes a straight line, from which a rate can be ascertained. The classical graph is obtained:-



1) Lag phase. Here the cells are adapting to the new environment. If a population of cells is introduced into a different medium, then time is needed to synthesise the various enzymes to metabolise. If the cells are placed into the same medium, then the expression of genetic information is already present. The required enzymes are available to the bacteria immediately, and the lag is

shortest. The phase of growth which the transferred cells were in, is also important. If the transfer consists of cells which have been left, and are probably in the death phase, perhaps no growth will proceed at all. If lag or stationary phase cells are used, a reasonably long lag will be encountered. The shortest lag is obtained using exponentially growing cells, transferred to the same medium.

2) Exponential or logarithmic growth phase. As cells come out of the lag phase, a phenomenom known as synchronous growth can occur. This is where all the cells divide at the same time, producing a false rate. This transient condition (which can be created and maintained experimentally) lasts only a couple of generations. Asynchronous growth now proceeds, and the cells grow exponentially or logarithmically.

3) Stationary phase. As the population grows, the medium constituents are used up, some minerals quicker than others. Eventually at least one will reach a limiting concentration, which is unable to support growth. The cells stop dividing. Alternatively, toxic waste products can accumulate, which will have the same effect. Thus the situation arises whereby some of the cells have stopped dividing, and an equal number continue to do so. The cell population apparently remains stationary.

4) Death phase. This happens when the number of cells growing is overtaken by those which can no longer grow. The cells which have not grown for some time start to die, and lysis occurs. These cells are irretrievably lost to the population, even after transfer to fresh medium. The overall effect on the population is to decrease cell number, the graph is seen to fall.

From this basic standard curve we can get the growth rate and doubling time. The **specific growth rate** is the differential:-

$$\frac{dx}{dt} = \mu$$

x = ln cell number or optical density

μ = specific growth rate

t = time (hours)

doubling time, $t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$

Both t_d and μ are expressed in hours. To work out μ and t_d, a growth curve must be created. Several techniques to analyse growth can be used:-

(a) Optical density (turbidity or absorbance measurements).

(b) Total cell counts.

(c) Viable cell count (only growing cells assayed).

(d) Dry weight (increase in biomass).

(a) Optical densities. This is the manner in which the majority of experimental μ values were created. The biggest advantage this has over **(b)-(d)** is speed. How are optical densities (or turbidity) changes detected? If a clear medium is inoculated with bacteria, eventually it will become cloudy. The extent and speed at which the culture becomes turbid, depends on the organism and the medium. The situation is made more difficult by the fact that until a good density is reached, the culture remains "clear". Usually $1*10^6$ bacterial cells/ml are needed before any visual change in turbidity is detected. "Clear" water, for example, is not at all "clear", when viewed microscopically.

Two types of instrument are used to detect changes in turbidity. The first, a **spectrophotometer** (using the principle of absorptiometry), measures the light transmitted through the sample. The second, a **nephelometer**, measures light scattered by the cells. In all cases in this present research, spectrophotometry was used. The machines used were **Baush and Lomb/Milton Roy Spectronic 20/20Ds** and an **LKB** one. The spectrophotometers used had a sample chamber with a light path of 1cm. The machine was first zeroed by using uninoculated medium. Thus at a suitable wavelength (660nm), the transmitted light was set to 100%, or zero absorbance. Samples of

cells progressively "absorb" more and more light, as the culture develops. The "absorption" (or deflection) of light by the cells was either read as a decrease in % transmittance, or an increase in absorbance. The wavelength (λ) used, 660nm, was entirely suitable to measure absorbance. Various workers have used wavelengths from 420 - 660nm. When a scan of blank medium was made, then the yellow colour absorbs strongly at 350nm. The range >400nm was suitable. The exact choice needed particular investigation, 660nm was suitable in this case. The changes in transmitted light were then measured using a photocell. The machines were read manually, as samples were collected from the reactor, but on-line measurement of absorbance using a chart recorder was also used. In order to do this, a flow-through cell was put into the spectrophotometer. Culture was pumped from the reactor, through the flow-through cell and back again. Providing the culture did not become too dense, or contains excess air bubbles, satisfactory results can be obtained. In practice, this was useful in measuring the exponential growth rate, in the earliest continuous culture experiments. Once the culture had grown sufficiently well in batch, the cell density became too high. Eventually, as the dilution rate was increased, on-line readings resumed. In cases when readings went off-scale, the culture was accurately diluted, as appropriate. Providing this was done carefully, samples were read manually and extrapolated by the dilution

factor. The **LKB** machine was able to handle denser cultures than the **Spectronic 20/20Ds**, before it too needed diluted samples.

Optical density, particularly absorbancies, represent a rapid way of assessing the growth of a culture. This is especially so when done on-line to a computer, which can carry out the mathematical analysis to generate μ.

Section C.1.1.2

(b) Total cell counts. The previous measure of growth can be corroborated by the result of cell counting. Total cell counts were determined manually, using a microscope. A special counting chamber, or **haemocytometer**, was used to count the cells. This comprised a calibrated grid on a special microscopic slide. The grid is sunk into the slide, to provide a specific depth, which is achieved by careful placement of the coverslip. Due to the thickness of both the slide and the coverslip, and practicality, the slide is viewed under 400X magnification. The 40X (phase-contrast) objective lens image is enlarged 10X by the eye-piece lens. 25 large squares are present on the grid, each of which is sub-divided into 16 squares. The area of each smaller square is $0.0025mm^2$, with a depth of 0.02mm. The total volume is therefore $5*10^{-5}mm^3$, or $2*10^{-7}ml$. Sufficiently dilute samples were placed into the well of the counting chamber, and direct visual

counting was carried out. This was a very lengthy, not to mention eye-straining and tedious, procedure. Ideally, 600 cells should be counted, and averaged per small square. From this, the number of cells/ml is calculated. The **ln cells/ml** versus **time** is plotted to give the growth curve, once more. The μ and t_d values are deduced, and compared to the previous method's answer. This technique has several problems: firstly accurate dilutions must be prepared to minimise error, and it is so tedious to do. An alternative method could be to use a **Coulter counter**, which measures particles of a defined range. This might, however, include "foreign" particles (not cellular material). Samples for total cell counts can be stored at $4^{o}C$, with a drop of concentrated formaldehyde. This prevents further growth, death or significant cell lysis.

Section C.1.1.3

(c) Viable cell counts. Viable cell counts have an advantage over the total cell counts, in that they only detect live cells. For a growth rate, this is obviously more accurate. There are problems associated with it, not least is the time needed to carry it out. To prepare viable cell counts, agar plates are used, onto which serial dilutions of samples are placed. These dilutions, usually $1*10^{6}$ to $1*10^{11}$, are performed in sterile saline solution (5%). The saline helps prevent too much

further growth, and thus invalidating the results. The agar plates are sectored, and then a drop (0.02ml) of each dilution is placed onto the relevant section. Once these drops have dried onto the surface, each plate of samples is incubated at 34°C overnight. The following day, the colonies are counted (>30, <200). The various dilutions should give equivalent results. This technique relies on the principle that **each cell will give rise to one individual colony**. Where two cells are adjacent, or on top of each other, then colonies could merge and produce spurious results. These problems, along with preparing **sterile** dilutions, if accounted for, enable a growth curve to be obtained. Once again the result can be compared to the earlier determinations. With a shake-flask experiment lasting up to 8 hours, hourly sampling meant a lot of preparatory work. For the fed-batch 50-hour and continuous culture experiments, it is not a practical proposition. Measurement of ***Alcaligenes latus*** using this technique would probably not be possible. The organism is sensitive to 5% NaCl in nutrient agar, so saline dilutions would be impossible. Using sterile water could lead to osmotic problems, leading to lysis in extreme cases. This would reduce the viable cell count, and thus the growth rate figure.

Section C.1.1.4

(d) Dry weight measurements. Samples for dry weight have to usually include up to 50mg of cells, in order to

minimise error. In the later stages of **PHB** accumulation, at 10-15g/l, this would only require up to 5ml of cells. For the earliest stages of growth, much more material would be required. During a long experiment of relatively small initial volume (750-850ml in a 1l reactor), the amount of sample required would be prohibitive. Not only that, dilution errors would then be introduced, as less culture would receive the same pH control. As dilute alkali was introduced, the culture would be diluted by removal of large samples. This would lower the growth rate figure. Typically when these experiments were carried out, the sample initially yielded less than 50mg of dried cells, and was therefore less accurate.

The actual process of recovering dry weight samples is done using vacuum filtration. Whatman cellulose nitrate filters (with a pore size of 0.2μm) were used. The supernatant recovered was used for glucose and ammonia determination, which was another practical way of cutting down sample volume. The dry weight is calculated by having pre-weighed filters, several of which will remain unused. A medium blank was filtered, which had no discernible weight. After drying overnight at 105°C, the filters are weighed (having reached ambient temperature in 0.5 hours). Including several unused filters is a safeguard against the filters losing weight themselves. The weights were plotted versus time, and a growth rate figure can be obtained. The weight associated with the

exponentially growing cells was too little to accurately determine a growth rate. To do so would have required larger samples. The purpose of the plot of weight of cells versus time was used as an indication of polymer storage. This occurs under nutrient limitation, and weight accumulation during the stationary (or storage) phase is attributable wholely to **PHB**. Thus, weight determination was used as a rapid indication of **PHB** quantity, in this example.

An alternative to using vacuum filtration is to dry-off 10ml of culture in predried test-tubes, weighed accurately. This provides a very satisfactory procedure, and was conducted at **ICI** as part of the "CASE" award industrial session.

In the statistical analysis, found in **Section C.4** of this Chapter, the comparison of different growth rates is detailed. Different media were selected from the literature, and the growth rate of each was ascertained. These rates were statistically compared, in order to select a suitable medium. They were also useful to check the suitability of scale-up, from shake-flask to 1/2l reactors, and ultimately to a 16l reactor. The result of this is found in **Chapter 5.**

Section C.2

Techniques for analysis and evaluation of growth.

Section C.2.1. The test for Protein.

The determination of cellular protein is based on the method of **Bradford (1976[87])**.

100mg of **Acid Blue 90** (also known as **Commassie Brilliant Blue G-250**, or **Page Blue "Electran" G-90**) was dissolved in 50ml of 95% ethanol. To this, 100ml of 85% orthophosphoric acid was added. The solution was diluted to 1l using deionised water. This is the **Protein Reagent**.

Bovine Serum Albumin (BSA) was made up to 100mg/100ml stock solution, 100µg/0.1ml sample. A series of 0-100µg solutions were prepared, using the following buffer: 30ml of 0.2M KOH added to 50ml of 0.2M KH_2PO_4, mixed together and made up to 100ml. 0.1ml of each of the series was pipetted into duplicate (or triplicate) tubes. 5ml of **protein reagent** solution was then added. The tubes were shaken and incubated at room temperature for five minutes. The stability of the protein-dye complex, is such that analysis may take place 5-20 minutes after adding the reagent. The colour develops in two minutes and breaks down after one hour. The samples were read in a **spectrophotometer** (Spectronic 20/20D - Bausch and Lomb/Milton Roy respectively) at a λ of 595nm, using

the appropriate filter and phototube. The cuvettes have a 1cm ø. Using this method, a protein standard curve was formed. When samples were zeroed against the blank one (0µg protein), the curve was statistically linear. The correlation coefficient (r) for the standard curve was always in excess of 0.95, showing good linearity. The standard curve for 0-100µg protein (**BSA**) versus absorbance is found on **Figure A1**, in the **Appendix Section**.

Samples from the vessel (for protein testing) were prepared using the following procedure. 1ml of cell sample was added to 1ml of 1M NaOH in a test-tube. The contents were mixed, and placed in a boiling water bath for five minutes. The alkali disrupts the cells, which allowed protein to be released. Once cool, the samples were shaken ready to take a 0.1ml aliquot for testing. Duplicates of each sample were put into test-tubes and 5ml of **protein reagent** was added. The test also included a couple of known **protein standards**, and a blank. The standards were used to check that the reagents are satisfactory. They should also closely agree with the values on the standard curve. The range 0-100µg/0.1ml is equivalent to 1g/l protein. The experimental samples were read as before, against a blank (at 595nm in the spectrophotometer). The values were then converted to the g/l protein present, averaging duplicates. The averaged value was then finally doubled, to account for the

dilution on extracting the protein (1:1 cells:alkali). The resulting data was then put on to a graph, either manually or using a computer package.

Section C.2.2. - The test for Ammonium Sulphate.

The determination of supernatant ammonium sulphate was based on the method of **Srienc, et al (1983[57])**, following the **Berthelot reaction.** In the majority of experiments conducted in this work, the limiting nutrient was nitrogen. The nitrogen source for the bacteria was in the form of $(NH_4)_2SO_4$, usually several g/l to start with. This progressively declined as the bacteria metabolise and grow, to the extent that available supernatant nitrogen was removed. When **one** of the available nutrients becomes limiting, the bacteria cease to grow, and enter the stationary phase. This is the stage when **PHB** is accumulated. Thus measurement of nitrogen is necessary in order to gauge when to begin to feed excess carbon, to promote **PHB** production.

The following two solutions were used to test for ammonium sulphate. **Solution A**: 10g/l phenol and 10mg/l sodium nitroprusside ($Na_2[Fe(CN)_5NO].2H_2O$). **Solution B**: 90g/l $Na_2HPO_4.12H_2O$, 6/g/l NaOH and 10ml/l NaOCl. 2ml of **Solution A** was mixed with 0.1ml of sample, and then 2ml of **Solution B** was added. The resultant mixture was incubated at 37°C for 30 minutes. The samples were read,

versus a reagent blank (no NH_4^+) in a **spectrophotometer** at a λ of 630nm. 1cm $\varnothing$ cuvettes were used, along with the correct phototube and filter.

The **Standard Ammonium Solution** was made up to 0.4g/l, (1/10 of the medium concentration being used at the time). 0.4g/l $(NH_4)_2SO_4$ was used as the stock solution, as this concentration gave a reasonable range with the spectrophotometer (Spectronic 20 or 20D). A series of 0-0.4g/l solutions was prepared, and into duplicate (at least) test-tubes, 0.1ml of these solutions were placed. 2ml of **Solution A** is added, followed by 2ml of **Solution B**. Once the tubes had been incubated and read, versus the blank, a standard curve was created. This curve was again statistically linear, the correlation coefficient, (r), was always >0.95. The standard curve for 0-0.4g/l **Ammonium sulphate** was shown on **Figure A2**, in the **Appendix Section.**

Preparation of samples from the vessel was somewhat unusual, and arose from an operational problem. At first, experiments were carried out in 1l vessels, with an initial working volume of 850ml. With batch and fed-batch work, continued sampling can result in operational difficulties. A set amount of culture must remain to immerse the pH, O_2 and temperature probes, and to avoid burning out the cartridge heater probe. Thus, when sampling for dry weights, using vacuum filtration, the

supernatant was collected. This was used for soluble nutrient determinations, such as carbon, nitrogen etc. Samples were diluted appropriately to bring them onto the range used in the test (which meant a 20X dilution for 4g/l media, giving a 0.2g/l solution). Once again, duplicate samples were tested, along with known **ammonium standards**, against a reagent blank (0.1ml water). The resultant values were then converted into g/l $(NH_4)_2SO_4$, taking into account averaged duplicates and the dilution factor. The data was then put into graph form using a computer package.

Section C.2.3. - The test for Glucose

The determination of supernatant glucose was based on the method of **Dubois, et al (1956[88])**. During the initial stages of batch growth of the bacteria, metabolism is concerned with replication - cell division proceeds. With exponentially growing cells, where there are no limiting nutrients, **PHB** formation is minimal. Typically it would amount to about 10% of cell dry weight. Under conditions of physiological stress, such as would occur during nutrient limitation (with the exception of Carbon) **PHB** is formed. This now takes over from cell division. This switch in metabolism is used to promote large levels of polymer storage (up to 70-95% of cell dry weight). The measurement of nitrogen, as described previously, indicates the stage at which

glucose can be fed. This point coincides with a drop in the rate of increase in readings of optical density and cell counts. Once glucose feeding starts, a check for incorrect feed rates would be carried out, with a steady increase indicating the end of storage. Faster methods for checking glucose are available, whereby automatic enzyme-reagent machines give instant readings. This method, of Dubois, et al, is more than adequate for giving accurate results. From sampling to reading takes as little as 40 minutes.

Two solutions were used to test for glucose. **Solution A**: 5% Phenol (5mg/100ml), **Solution B**: Concentrated H_2SO_4. A **stock glucose solution** was also prepared, to create a standard curve. This was made up to 100µg/ml, or 10mg/100ml. 1ml of a series of 0-100µg/ml **glucose standards** were placed into duplicate thick-walled test-tubes. To these, 1ml of **Solution A** was added, and the tubes were shaken. Using extreme care, 5ml of **Solution B** was added. This is done reasonably fast, and in a fume cupboard, preferably, due to the acid fumes. If acid is added in only moderately quickly, then the reaction can be violent. When added fast, the acid dilutes the reaction sufficiently to be less dramatic. Once the acid was added, the contents were mixed and left for 10 minutes to cool down. The reaction is a very exothermic one, and immediately an orange colour is formed, if sugar is present. Once cool, the

tubes were incubated for at least 15 minutes at 30°C. The colour remains stable, however, for several hours. The samples were read against a deionised water blank. The **reagent blank** (0µg glucose) value was subtracted from all the other readings. Samples are read in a **spectrophotometer** at 488nm for glucose, using the correct filter and phototube. The standard curve produced was once again statistically linear, the correlation coefficient (r) was >0.95. The standard curve of 0-100µg/ml **glucose** versus absorbance was found in the **Appendix Section, Figure A3.** Actual experimental samples were prepared from the vacuum filtration supernatant solutions. These were also used to measure ammonium. They were diluted appropriately (in this case a 250X dilution for 20g/l media, giving an 80µg/ml solution) and duplicate samples were prepared. 1ml **Solution A**, and 5ml **Solution B** are added. The tubes are incubated and read as before. Using **glucose standards**, to check the procedure, the values for glucose were obtained. These were averaged, multiplied up to account for the dilution, and then put into graph form.

Section 2.4. - The Test for PHB

Two determinations of **PHB** were carried out. Firstly, a gas chromatographic method as devised by **Braunegg, et al (1978[55])**. Secondly, an enzymic method described by **Johnson (1986[60])** and **Mann (1985[59])**, both of **ICI**

Biological Products, Billingham.

The **Braunegg** method **(1978[55])** utilises a double FID (flame ionisation detector) gas chromatograph. This was a Pye 104 in this case. The glass column used was 275cm (nine feet) long, with a 6.5mm (1/4 inch) external diameter (ø), and a 2mm internal ø. This was filled with 2% **Reoplex 400**, on **Chromosorb GAW 60-80m.** The chromatograph was connected to a chart-recorder via a Pye DP88 computing integrator. Nitrogen, at 30 ml/minute was the carrier gas, with hydrogen and oxygen for the flame detector. The chromatograph was programmed to run from 70-150°C (at 8°C/minute steps), to elute the various injectants. The **standard references** of pure **PHB** were prepared. 1-20mg pure **PHB** samples were dissolved in 2ml of acidified methanol (3% v/v H_2SO_4 content). 2ml of chloroform was added, the solutions were then incubated at 100°C for four hours. This mixture requires glass test-tubes with srew-capped PTFE lined lids. When cool, 1ml of deionised water was added, and the resultant mixture was shaken for 10 minutes.

When **PHB** is degraded in acidified methanol to crotonic acid methyl esters, the water addition drives it into the chloroform phase. 2µl of the organic layer, which forms on settling, was injected onto the column. The integrator works out the peak areas for $CHCl_3$ and **PHB.** In addition, a benzoic acid standard was used,

prepared in the normal way, to check the column. The retention times for $CHCl_3$, **PHB** and benzoic acid were 30 seconds, 6 and 8 minutes after injection, respectively. $CHCl_3$ comes off immediately at around 90°C, **PHB** at around 125°C and benzoic acid at about 140°C. The standard curve of $\log_e$(ln)[area of $CHCl_3$ peak/ area of **PHB** peak] versus **PHB** is found on **Figure A4**, in the **Appendix Section**. It was not linear, but a gradual curve when plotted in this way. When the area of the **PHB** peak was plotted against **PHB standards**, then a linear relationship was found. Use of $\log_e$ $CHCl_3$/**PHB** peak areas against **PHB** quantity was necessary to account for experimental errors. Injecting 2µl precisely each time, unless automated, was not feasible. Therefore, for a set value of **PHB** in the sample, 3 **PHB** peak areas could have widely different results, caused by injection volume error. By comparing the $CHCl_3$/**PHB** peak areas, even if the volume used is different, the ratios will be the same for each **PHB** sample. Sample ratios are back calculated to readings of **PHB** by reading off the curve.

To prepare unknown samples for injection, the cells were dried. The weighed samples were put into the test-tubes, and incubated with the solvents for four hours at 100°C, as before. After cooling, adding water and shaking, the samples were left to settle. The organic phase was then kept for analysis. 2µl of sample was injected, and a ratio of $CHCl_3$/**PHB** peak areas was

obtained from the integrator readings. This converted into a weight of **PHB**, mg/2ml sample. It was sometimes necessary to dilute the samples in $CHCl_3$ to get a suitable peak height. Once several values were obtained, (and the standard error of the mean was below 5%) the average value was taken. All the samples were treated likewise, and then the data was put onto graph form.

This technique was described as being rapid, and having a high accuracy and reproducibility. However, in practice two problems were encountered. These led to the abandoning of this technique for the remainder of the research. The first problem was that the solvent mixture attacks the lids of the test-tubes. This could have been overcome by choosing more compatible, robust lids. Too many samples were lost due to this. Secondly, the integrator failed repeatedly throughout the runs. After considerable time had been expended on this, it was decided to leave it. The combination of the two, along with notification of another technique, led to abandoning this method.

The current method was obtained from **ICI, Biological Products**. This was done through the industrial session at **ICI**, as part of the **CASE Award**. The technique **(Mann, 1985**[59], and **Johnson, 1986**[60]) involves hydrolysis of the **PHB polymer** to D-3-hydroxybutyrate. In the presence of nicotinamide adenine dinucleotide (NAD) coenzyme,

D-3-hydroxybutyrate dehydrogenase enzyme (3-HDBH) oxidises hydroxybutyrate into acetoacetate. The reaction is thermodynamically favourable with respect to the reverse reaction, however. Under appropriate conditions, co-reduction of NAD to NADH is encouraged. NADH absorbs light at a wavelength (λ) of 340nm, and is then measured spectrophotometrically. The formation of NADH is therefore an indicator of initial **PHB** level. The more **PHB**, the more NADH formed, and thus the more absorbance.

The analysis and determination of polymer content of cells was performed (in addition to using known **PHB** samples) in an autoanalyser. This records the maximum absorption over a 40-60 minute period. Cell material was either freeze-dried or heat-dried, and then weighed out accurately. Using the standards, the analyser determines the weight of **PHB** in the sample present. A % **PHB** content of cell dry weight was then determined (**PHB** weight/ weight of the sample X100% gives the % content). This technique is an **ICI** one, which is protected by suitable patents. This enables protection, and requires their permission to use it. The accuracy and reproducibility of the technique was excellent, and very satisfactory results were obtained. Once again, data from the samples (either as g/l **PHB** or % **PHB** content versus time or dilution rate) was plotted on a computer generated graph.

Section C.3. - Data Presentation, the visual picture.

All the experimental parameters were put into a graph plotting program, which was devised from an earlier version by **Mr Neal Smith**, a former colleague within the department. The system is set up to display a menu, from which the relevant program is selected. Firstly a data input program is used, which allows the simultaneous input of 1x and 3y values, on the same axis. An editing facility allows for input error, and when complete, the data is stored as a named file on a floppy disc. Selecting the graph program, the data file is printed onto a conventional printer. The computers used to run this system were the **BBC Model B** and **Master** microcomputers. The graph program also allows you to have a "data-echo" (the data points are stated below the relevant graph). Combinations of data from experiments are presented as:-

Graph 1 - Log_e (ln) absorbance of cell culture (optical density) measured at 660nm, versus time (hours).

Graph 2 - Glucose, ammonium sulphate concentration and dry weight (g/l) vs time (hours).

Graph 3 - Protein concentration (g/l) vs time (hours).

Graph 4 - Log_e (ln) total cell counts/ml vs time (hours).

Graph 5 - **PHB** concentration (g/l) vs time (hours).

Graph 6 - Average cell weight (g/l) of $1*10^{10}$ cells/ml vs time (hours), **PHB** concentration (g/l) of $1*10^{10}$ cells/ml

vs time (hours).

Graph 7 - **PHB** storage rate (g/l/hour) vs % **PHB** accumulated (% of cell dry weight).

Graph 8 - Aeration (dissolved oxygen concentration, relative to 100% air saturated solution) and rpm of stirrer vs time (hours).

Section C.3.1. - Explanation of graphs.

Graphs 1-5 and 8 are merely straight forward plots of data or $\log_e$ data. Graph 6 is used to assess what the cells are doing with regard to their average weight. If an increase in weight/cell is shown, the **PHB** plot is used to gauge whether the weight increase is a factor of growth or storage. Graph 7 is a means to compare our work with previous works. At a set time into the experiment, a set **PHB** concentration is seen. By looking at the formation of **PHB** since the last data point, it is possible to get a **PHB** accumulation rate between these two points. Thus, the percentage and the resultant rate of accumulation is plotted. This is important to assess this bacterium's efficiency at storing **and** tolerating high levels of **PHB**.

The combination of graphs 1-5 could be done differently, it is usually done in this way due to the order in which the analysis is completed. Whilst a 50-hour fed-batch (or a continuous culture) experiment is

underway, there is little time to do more than take readings (optical density, aeration etc.) or prepare other samples. Cell count samples are stored at $4^{o}C$ (roughly 1ml of sample is treated with one drop of concentrated formaldehyde). This prevents cellular lysis and allows satisfactory results to be obtained, even after cold storage. Protein samples are collected and frozen, experimentally it has been shown not to affect the protein content. Glucose and ammonium sulphate samples are also stored frozen (collected as supernatant from the dry cell weight filtration), and again are not harmed by freezing. **PHB** samples are taken from the protein sample (several mls). In fact, freezing whole cells can disrupt the cell wall, making protein and **PHB** extraction for analysis more easy.

The graphs visually show what is happening in the bioreactors during experimentation. One individual plot considered in isolation is of relatively little use, errors in preparation of samples can lead to further problems in analysis. Considered as a whole, the parameters are of more use collectively, errors in one are taken account of. The physiology of the cells is now seen properly, further analysis and conclusions can now be derived.

Graphs of standard curves, which require statistical analysis, are done using the "BBC Model B" microcomputer,

and the data plotting programs, used in the **Polytechnic.** Statistical analysis usually takes the form of best-fit figures, and determining the correlation coefficient, **r**, of the data points. If a straight line is drawn, the coefficient r will equal 1 (+ve or -ve depending on the slope). The larger the deviation, the less accurate is the data line. Usually to consider a line straight, statistically for biology, 5% tolerance is allowable. The coefficient r must therefore not be lower than 0.95 (or more than -0.95 for curves sloping the opposite way). The standard curves are found in the **Appendix Section, Figures A1 - A5,** whilst data graphs are depicted in the **Results** section.

Section C.4. - Statistical Analysis - Significance testing for growth on various media.

When the project was started, a survey of experimental methods was undertaken, to find a suitable growth medium with which to progress. "Suitable" at this stage meant a medium which allowed the fastest growth rate; the derivation of a μ_{max} figure. Cells were encouraged to grow as fast as possible, to provide the highest level of biomass for the secondary stage of the process (polymer storage). However, the fastest growth of cells need not lead to the highest cell density. This, on reflection is fairly logical, and was an oversight. The intention was to get a high cell density in as short a

time as possible, which would then lead to a higher **PHB** density in the end.

With this aim in mind, several media were chosen. The different media (five initially) were used with batch culture shake-flasks, in triplicate, and frequently repeated. The composition of the five media (with several later ones, and a comparative strain) was given earlier in this chapter (**Section B.1, Table 3**). At 1/2 - 3/4 hourly intervals the cultures were removed from the shaker, and aseptically sampled. These were read for optical density (or absorbance), when the natural log of absorbance was plotted (ln Abs. 660nm). This produced the normal growth curve, from which the growth rate was determined. The exponential growth (linear) part of the curve provided the specific growth rate, μ. Mathematically this is expressed as:

$$\frac{\delta x}{\delta t} = \mu,$$

x = cells or optical density (absorbance or turbidity), mass, viable cell count etc.

t = time (hours).

μ = specific growth rate (hrs)

Thus, over the linear part of the graph (during exponential growth), μ is equal to the change in y-axis over the x-axis. The full explanation of the determination of μ was given in **Section C.1.1.** The

initial specific growth rate was used for direct comparison between different media in this case. In addition, other information was gleaned. On it's own, the figure is used as a test of the microbes stability, particularly if it is a newly created strain. Any reversion or further mutation could lead to a change in the initial specific growth rate. Secondly, it was used to gauge the time needed to increase a cell population to a suitable density. The cell doubling time (t_d, or how long it takes the population to double in size, 1 cell-2, 2-4, 4-8, 8-16, etc.) provided this information, and was calculated like so:

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$$ (t_d is also expressed in hours).

The best medium, under these requirements, had the smallest doubling time and the fastest growth rate. The actual determination of the best medium was done using the μ values obtained, there were at least three and normally six values. The triplicated and repeated experimental values were checked for deviation, which is to say they had to be within 5% of the mean standard error. Consider the following example:

Medium 1, μ values of a,b,c,d,e and f hours. The mean of medium 1 values is required, along with variation about the mean.

$$\bar{x} = \frac{a+b+c+d+e+f}{n}$$

$\bar{x}$ = the mean.

n = number of samples.

a-f = μ values.

Standard error = $\sqrt{\sigma^2/n}$

(SE)

σ^2 = variance about the mean.

σ = variation.

Standard error of the mean = $\frac{SE}{\bar{x}} * 100\% = \_\_\_\_\_\%$.

$SE_{(M)}\%$

If the $SE_{(M)}\%$ for the μ values is below 5%, then the values are all indicative of the same medium. To gauge significant differences between the media, the Student's t test is used.

$$t = \frac{(\bar{x}_2 - \bar{x}_1)}{\sqrt{\left\{\left(\frac{(\Sigma(x_1-\bar{x}_1)^2 + \Sigma(x_2-\bar{x}_2)^2)}{(n_1+n_2-2)}\right) * \frac{(n_1+n_2)}{(n_1*n_2)}\right\}}}$$

Once the value of t is obtained, using n_1+n_2-2 degrees of freedom, a table of t values is consulted (**Meddis, 1975**[89], p45). If the experimental t value is higher than the one in the table, at the 5% significance level, then there is a significant difference between the two media. Conversely, a t value below that of the tabulated one indicates no significant difference.

Medium 1	Medium 2
$\mu_1 = 0.30$	$\mu_2 = 0.51$
0.31	0.52
0.32	0.53
$n = 3$	$n = 3$
$\bar{x}_1 = 0.31$	$\bar{x}_2 = 0.52$
$\Sigma x_1 = 0.93$	$\Sigma x_2 = 1.56$
$\Sigma x_1^2 = 0.2885$	$\Sigma x_2^2 = 0.8114$
$(\Sigma x_1)^2 = 0.8649$	$(\Sigma x_2)^2 = 2.4336$
$\Sigma (x_1 - \bar{x}_1)^2 =$	$\Sigma (x_2 - \bar{x}_2)^2 =$
$\Sigma x_1^2 - \frac{(\Sigma x_1)^2}{n} =$	$\Sigma x_2^2 - \frac{(\Sigma x_2)^2}{n} =$
$= 0.2885 - (0.8649/3)$	$= 0.814 - (2.4336/3)$
$= 0.2885 - 0.2883$	$= 0.8114 - 0.8112$
$= 0.0002$	$= 0.0002$

$$t = \frac{0.52 - 0.31}{\sqrt{\left(\left(\frac{(0.0002 + 0.0002)}{4}\right) * \frac{6}{9}\right)}} = \frac{0.21}{\sqrt{\left(\frac{(0.0004)}{4}\right) * \frac{2}{3}}} =$$

$0.21/\sqrt{0.0000667} = 0.21/0.008165 = 25.7196$

μ = initial specific growth rates, h^{-1}.

t_d = cell doubling time, h^{-1}.

$\bar{x}$ = mean.

Σx = sum of the μ values.

Σx^2 = sum of (μ values squared).

$(\Sigma x)^2$ = (sum of μ values) squared.

n = number of μ values.

t = Student's t value.

For 4 degrees of freedom, (n_1+n_2-2 = 4) the t value is far in excess of the tabulated one. Thus there is a significant difference between the media. This is an exaggerated case, medium 2 was obviously better without actually doing the statistics! This produces results which can be put onto the following form of table:

Medium Number	1	2	3	4
5				
4				
3				
2				

Into each box, the actual t value (of the compared media) is inserted, along with the degrees of freedom and an arrowed indication of the better medium.

For example, for medium 1 vs. 2, the result is:-

Medium	1
2	← t=25.72 dof=4

(where dof = degrees of freedom).

With this example, medium 2 is best, hence the arrow points in favour of 2.

From this, it can be seen that a ranking system can be devised. At a glance, it can be seen which medium or media are best. The values obtained are given in **Chapter 5, Section A.2.**

Section D

Harvesting and extracting the cell material and polymer. Analytical techniques to assess polymer quality.

Section D.1 - Harvesting.

At the end of an experiment (conducted with batch, fed-batch or continuous culture) the reactors were drained. The ex-reactor cell volume was measured and noted. The reactor contents were acidified to pH 5.0, to prevent further reaction (with the exception of the 250ml shake-flasks), leading to cell death and lysis. This would release enzymes, but the acid pH would deactivate the enzymes and prevent degradation of the polymer. This pH was sufficiently "mild", however, not to attack the polymer itself. The cell solution was stored at $4^{o}C$ until required.

Extraction took the following form. The cell solution volume was measured, and then the cells were centrifuged. This took place in an **MSE 18** refrigerated centrifuge, at 6000 rpm (for 15 minutes, at $4^{o}C$). Two procedures were then followed for extraction.

Section D.1.1. - (i) Solvent route.

The centrifuged cells were dried at $105^{o}C$, overnight. This was the method of **Holmes, et al, (1980[64])**. The dried

cell powder was placed into a round-bottomed flask, to which 500ml of methanol was added. The mixture was refluxed (with anti-bumping granules) at 65°C for five minutes. The lipid portion of the cell was leached out into the methanol, which goes yellow as a result. **PHB**, being insoluble in methanol, remained in the now "leaky" cells. These were separated from the methanol/lipid phase by vacuum filtration. Whatman PTFE backed filters were used, which have a 0.2µm pore size (using Pyrex "Millipore" glassware). The lipid-free cells were dried and reground, and then refluxed for 15 minutes in chloroform, at 61°C. The hot chloroform extracted the **PHB** into solution, and the liquid phase was separated by filtration. Whatman cellulose nitrate filters were used, with the same pore size as before. The **PHB**-containing liquor was poured into a rapidly stirred solution of 4:1 methanol:water mixture. The chloroform releases the **PHB**, which flocculates out of solution. The solution was filtered to recover the **PHB**, and this was then dried overnight at 105°C. After allowing it to reach ambient temperature (for 0.5 hour), it was weighed. Knowing the cell dry weight, the volume of cells harvested and the **PHB** content, the recovery efficiency was assessed. If the cells contained 75% **PHB**, at a dry weight of 15g/l, and a volume of 500ml was used, then **PHB** recovery should yield: 15/2 * 0.75 = 5.625g of **PHB**. If the recovery = 5.25g, then the system was 5.25/5.625 * 100% = 93.3% efficient, 6.667% was lost on extraction. The polymer was then used

for analysis or moulding.

Section D.1.2. - (ii) Enzymic extraction route.

The centrifuged cells were resuspended in the original volume of deionised water. They were homogenised to resuspend and were then treated:-

(a) First Digest. The resuspended cells were rapidly brought to 70°C (using live steam, controlled with **EXTREME CARE**), and then put onto a preheated magnetic stirrer base. It was kept agitated throughout, and the temperature was maintained using a thermocouple.

The extraction enzyme, **Optimase L600**, was weighed out to give a 0.5% weight/biomass ratio. ie Biomass at 50g/l cell dry weight needs 0.5% * 50g/l = 0.25g. The enzyme was premixed in about 10ml of cold water, and was then slowly added to the preheated, mixed culture solution. The pH was increased to 8-8.25 using 1M NaOH (4% solution). This was maintained by an autotitrator, pumping in OH^- when the pH probe detected a fall in pH. The solution remained under these conditions for two hours. After such time, the solution was centrifuged once more, and the supernatant discarded (or retained for N, P or S analysis).

(b) Second Digest. The centrifuged cells were

homogenised in their original volume of fresh deionised water. A detergent was then added to further disintegrate the non-**PHB** cell material. Enzymic treatment breaks open the cells, and attacks proteins and nucleic acids. The detergent solubilised these fragments, and the lipid present in cell walls (including the membrane around the **PHB** granules). The detergent **NP8** was added in a 1% weight/volume ratio. Thus, for 500ml of solution, 5g of detergent was needed. This was also premixed in about 10ml of cold water. The detergent was added to the solution, gradually. The cell suspension was brought to 70°C rapidly, and placed onto a magnetic stirrer base. The agitation was maintained for the duration of the second digestion. . The pH was adjusted to 7.0 using 5% HCl, and maintained by use of an autotitrator. The extraction lasts two hours. The solution was centrifuged again, in readiness for the final treatment.

(c) Peroxide Treatment. This final treatment was carried out at 80°C. The resuspended and homogenised cells were rapidly heated, and then kept agitated. H_2O_2 was added to a strength of 2 volumes of "130 volume" peroxide (39% solution). Thus for 500mls, we have 2/130 * 500 = ml of H_2O_2 required = 7.69ml/500ml culture. This was added to the heated cell solution, which was now not pH controlled. The incubation was allowed to proceed for one hour, when a further 2 volumes were added. After a total of 2 hours incubation, the suspension (which by now

was at a pH of 3.4 - 3.5) was boiled for 15 minutes (100°C).

(d) Recovery, by means of filtration, was done through a vacuum filter system. The filter cloth size was "N22", and the vacuum was maintained at 15-20psi. The time taken to filter through the suspension, to obtain a matt filter cake, was recorded. The polymer was transferred to a weigh-boat, and dried overnight at 105°C. The polymer weight was recorded the next day, after allowing 0.5 an hour to reach ambient temperature. The efficiency of extraction was assessed as in the first procedure. The dry polymer was collected for analysis.

For these particular experiments, procedure 1 was initially followed. Procedure 2, with variations, has been used in conjunction with the "CASE" award industrial sessions. This was done to assess various extraction techniques. In the later stages of the research, the second procedure detailed above was used.

Section D.2. - Analytical techniques to assess the quality of polymer formed by bacterial experimentation.

Several techniques were used:-

(a) Gel-permeation chromatography (GPC): this enabled the determination of polymer molecular weight.

(b) Differential scanning calorimetry (DSC): this enabled a unique profile of each polymer to be obtained.

(c) High performance liquid chromatography (HPLC): this was of use in quantitative analysis of percentage **HV** present in copolymers.

(d) Nuclear magnetic resonance (NMR): used for the same reason as (c) above.

(e) Melt-flow index (MFI): a characteristic index of each polymer was obtained, but copolymers can give problems.

(f) Various physical techniques: impact strength, load capability and stresses, brittleness, etc.

Section D.2.(a). - Gel-permeation chromatography.

This was a quick and accurate method to determine the distribution of molecular weight. The quantity of pure polymer required was only a few mg, so it was suitable even for initial bench-scale experiments. First the polymer was dissolved in a solvent. In the case of **PHB**, and **HV** containing copolymers, this was chloroform (heated slightly if necessary). The chloroform solution was introduced into the solvent stream of the column. The column was filled with porous glass beads or cross-linked polystyrene, for example. The pore-size and the packing material depends on the chosen analytical procedure being used (details are not given as samples were run for, not by the author of this thesis. The same applies to details of **HPLC**.). The dissolved polymer passed down through the

column, whereby the larger molecules were excluded from entering the pores. These eluted fastest, the smallest and mean-sized molecules were held in the pores for a variety of time. The emergence of the polymer eluted gives the molecular size variation. The analysis calculates the distribution (measured in Daltons) and gives the mean value. Typically for **PHB** the distribution was $1*10^4$ - $8*10^6$, with an average value of $1*10^6$. The exact values were given in the **Chapter 7, Section A.1.(c).(i).**

The molecular weight value is of use to an engineer, in choosing an appropriate polymer for a particular application. Generally, the highest molecular weight possible is required, as this would have the highest strength. A balance between viscosity increase (being a function of increasing molecular weight) and optimum strength needs to be struck, however. Choosing the highest molecular weight (to gain strength), would be little use if the polymer was now too viscous. The effect of increasing molecular weight (with a corresponding rise in viscosity) was shown dramatically with **MFI.**

Additionally, molecular weight values were used to assess the quality of the polymer. If an extraction procedure was so severe, giving only low molecular weight polymer, it would not be suitable. In the case of **PHB**

(which was biodegradable), if left stored wet microbial action would proceed. This would degrade the polymer, and thus **GPC** could assess the integrity of storage.

Section D.2.(b) - Differential scanning calorimetry.

This technique enables the detection of the melting and recrystallisation points of polymers. Each particular pure polymer will have it's own distinctive profile. Pure **PHB** has a characteristic melting point. This varies only if the molecular weight was significantly different between samples. For **HV** containing copolymers, the situation was different, **HV** monomers were put in the polymer chains in a random manner. This would give the polymer a heterogeneous nature, thus the same sample of copolymer could give a different **DSC** profile. The overall melting point of similar copolymers (the same **HV** content) was reasonably constant, however. Copolymers also lack a recrystallisation point, as opposed to pure **PHB**.

The quantity of polymer or cell material needed for analysis was again small, being 10 - 20mg. This was weighed to the nearest 0.01mg, and placed into a **DSC** sample pan. A lid was put onto the pan, which was then analysed using a reference sample (usually an empty pan). The changes in calorimetry were measured as the sample was taken from about 50 to 200^{o}C, and back down again. A **Perkin Elmer DSC 4** was used, which gave a printed

profile. The onset and regression, melting and crystallisation points were calculated. These figures were used for qualitative analysis of polymers. The melting and recrystallisation points of the polymers were detailed in Chapter 8. This technique was also useful as a quantitative test for copolymers containing **HV**. If a set of known copolymer standards were analysed using **DSC**, a calibration curve of melting point vs %**HV** was devised. Thus for any unknown **HV** containing sample, the melting point will give an estimate of **HV**. In practice, this was only suitable as a guide, further analysis was used to accurately determine %**HV**. The result of using **DSC** as a quantitative test was shown in **Chapter 7,Section A.1.(c).(ii)**, along with a discussion of the validity of using it in this manner.

Section D.2.(c) - High performance liquid chromatography.

Samples for **HPLC** were prepared in a similar way to enzymic analysis of **PHB** (see Section C earlier in this Chapter). This technique was also an **ICI** developed one. Unlike tests **(a)** and **(b)**, this requires comparatively large samples of polymer, 150mg. The test was a quantitative one for **HV** presence in copolymers. The polymer was degraded in perchloric acid, neutralised to remove protein, and then fed into the **HPLC**. In this instance, reverse phase **HPLC** was used. The sample was injected onto the column, where it elutes over one hour.

The peaks were integrated and a plot was recorded. The %**HV** was shown by peaks of **HV** and **HB** monomers. %**HV** equalled the area of the **HV** peak divided by the sum of the areas of both **HV** and **HB** monomers * 100%. **HPLC** was used to compare several quantitative methods for **HV** detection (such as **DSC** and **NMR**). The results are detailed in **Chapter 7, Section A.1.(c).(iii).**

Section D.2.(d) - Nuclear magnetic resonance.

This gave the best quantitative analysis of **HV** content of copolymers. Approximately 100mg of pure polymer was required for analysis, which was done at **The University of Newcastle.** The **NMR** machines used were of the 90 - 300MHz range, where the higher the frequency, the better the resolution. 1 sample was run on the **Polytechnic's JEOL 90MHz machine, by Mr Steven Reade.** Deuterated ('H) chloroform was used to dissolve the polymer which was then analysed. Peaks were formed corresponding to -CH, $-CH_2$ and $-CH_3$, of the **HV**, **HB** and **HV/HB** mixtures. Two measurements were performed:-

(i) The mol %HV using the methyl resonance:

$$\frac{\text{mol ratio of HV}}{\text{HB}} = \frac{\text{integral (HV) } CH_3}{\text{" (HB) } CH_3}$$

$$\text{mol \%HV} = \frac{\text{integral (HV) } CH_3}{\text{integral (HV) } CH_3 + \text{integral (HB) } CH_3} * 100\%$$

(ii) The mol %HV using the methylene resonance:

$$\text{mol ratio of } \frac{HV}{HV + HB} = \frac{\left\{\frac{\text{integral of (HV) } CH_3 \text{ groups.}}{3}\right\}}{\left\{\frac{\text{integral of (HB) } CH_3 + \text{(HV) } CH_3}{2}\right\}} * 100\%$$

Both were done, and were detailed in **Chapter 7, Section A.1.(c).(iv).**

Section D.2.(e) - Melt flow index.

This technique was a variation of the **capillary flow experiment.** The rate of extrusion of a polymer melt, was determined through a given capillary, in a defined piece of apparatus. A heated barrel (9.57mm ø) was connected to a capillary 0.8 mm long, and 0.209mm wide. Polymer (for **PHB** and copolymers, 5g was used) was packed into the barrel, which was maintained at 190°C. A piston was applied with a weight put onto it. After a few minutes, the force of the piston ejects the polymer through the hole. At set intervals, polymer extrudate was cut off and weighed. This gives an index for each particular polymer. The higher the molecular weight, the stronger the polymer

(cross-linking etc.). This in turn means increased viscosity and the greater the force needed to eject it, and vice versa. Thus it was used in quality control and qualitative analysis. The results were shown in **Chapter 7, Section A.1.(c).(v).**

Section D.2.(f) - Various physical tests were performed to analyse the impact strength, tensile strength, brittleness, etc of polymers. The polymer was cast into "closed-spanner" shapes, which were then set into a stress measuring machine. As the polymer was pulled apart, it firstly stretches, reaches it's elastic limit and then snaps. The degree of elasticity was governed by the polymer's physical characteristics. Pure **PHB** (or **PHV**) was very brittle, and had less impact strength. This would not be suitable to form large quantities of articles, therefore. Copolymer blends of various **HV** content, exhibit a range of properties, which enable specific practical application. Further discussion of the physical testing of the pure polymer and copolymer will not be given, as it was outside the scope of the present work. For more information, refer to references **75-78.** If further information on general polymer engineering was required (**MFI, GPC,** etc), refer to **McCrum, et al, (1988[90]).**

Chapter 5

Section A - Shake-flask experiments, determination of growth rates.

Section A.1.

The first objective of the research, was to get a suitable growth medium, with which to go on to examine **poly-β-hydroxybutyric acid (PHB)** accumulation. The organism which was selected to do this research, was ***Alcaligenes eutrophus*** **H/16 S301/C5**. This was supplied by **ICI Biological Products**, the industrial sponsors. Two other organisms were examined, albeit briefly. These were ***Alcaligenes eutrophus*** **H/16 S301/TRON**, the production strain, and ***Alcaligenes latus*** **NCIB 12189 (DSM 1123)**, as used by the Austrian researchers. **Table 3**, in **Chapter 4, Section B.1.**, describes nine media. Of these, there is one for ***Alcaligenes latus***, and a "one-off" for cultivating **C5** in continuous culture (used at **Bradford University**). For the purposes of this chapter, ***Alcaligenes eutrophus*** **C5** was grown on media 1-5, 10 and 11. ***Alcaligenes eutrophus*** **TRON** was grown on media 5, 10 and 11, by means of a comparison.

Section A.2. - Results of initial shake-flask runs.

The technique of growing shake-flasks (including

"seed"-flasks) was described in **Chapter 4, Section B.1.1.**, along with the method of determining a growth assessment. Subsequently, specific growth rates (μ) of the bacteria on each medium were spectroscopically determined. **Table 4** depicts the average specific growth rates (μ, hours) for triplicated shake-flasks of each medium examined. In addition, the table also includes the doubling times (t_d, hours), but only μ values were used to compare with previous research.

Table 4

Medium No.	C5 1	C5 2	C5 3	C5 4	C5 5	TRON 5	C5 10	TRON 10	C5 11	TRON 11
Specific growth rate (μ), h^{-1}	0.384	0.474	0.523	0.416	0.539	0.475	0.555	0.531	0.726	0.605
Doubling time (t_d), h^{-1}	1.80	1.46	1.33	1.67	1.29	1.46	1.25	1.31	0.95	1.145
Shake-flask sample size	6	12	6	3	3	3	3	2	2	2
Standard error of the mean $SE_{(M)}$%	2.6%	2.2%	2.1%	4.6%	0.5%	2.2%	1.7%	4.7%	0.7%	6.0%

The last three media described in the table above, gave somewhat problematical results. Triplicated flasks were run, as usual, but in each experiment, only two of the three flasks ran properly. On the last two occasions, the third flask failed to appreciably grow. In fact, the last experiment produced significantly dissimilar results, from two flasks! It is important to bear in mind, however, that the last five media described were run as comparative experiments only. These five

shake-flask experiments were not repeated due to time constraints. They were also not done until the fed-batch and continuous growth of ***Alcaligenes eutrophus*** had already taken place. Media 10 and 11 were taken from current **ICI** technology, after the initial medium characterisation had been dealt with. The significance of the comparative nature of these last experiments will be seen later.

Initially, five media were chosen, one of which was adapted from an earlier one. Media 1 and 2 were described in the patent of **Hughes and Richardson (1982[35])**, media 3 and 4 were described by **Holmes, Wright and Collins (1983[36])**. As can readily be seen from **Table 3, Chapter 4, Section B.1.**, the only difference between media 3 and 5, is the levels of $(NH_4)_2SO_4$ and the inclusion of specific trace elements (other than the level of available carbon). Why was medium 5 described as a separate entity?. **Repaske and Repaske (1976[46])** grew ***Alcaligenes eutrophus*** autotrophically, and very good growth was possible with the inclusion of **chromium (Cr), nickel (Ni), and cobalt (Co)**, as trace elements. To this end, and to provide a medium which supported only a limited amount of exponential growth, $(NH_4)_2SO_4$ was dropped to 3g/l, and the extra trace metals were included. By restricting the level of $(NH_4)_2SO_4$ (in a medium where nitrogen limitation would be used to promote **PHB** synthesis), exponential growth would take place for a

shorter time. The cells would grow, but for less time, as the available nitrogen would be depleted quicker. The effect of using these trace elements would be assessed, in a chemoheterotrophic regime. The lower level of nitrogen would not affect the growth rate, and it was hoped to see whether the trace elements would affect this also.

Friedrich, Friedrich and Bowien (1980[50]) describe how the use of certain substrates (predominantly carbon) increased the levels of autotrophic enzymes, during heterotrophic growth. Thus, the inclusion of trace elements to encourage autotrophic growth, might be useful in heterotrophically growing cells. The object of inclusion was not to promote conditions of autotrophy, and thereby increase the growth rate, but was done to ensure sufficient quantities of these trace elements were present. It may be the case that even highly pure chemicals (such as **BDH's AnalaR** reagents), may contain sufficient quantities of trace elements to satisfy the cells requirements. Experimentally, it was found that the inclusion of these trace elements (Cr, Ni and Co) did not significantly affect the growth rate. Indeed, purely by accident, an experiment was done in which these trace elements were added ten-times stronger than described in **Table 3**. This led to a 30% reduction in the growth rate, with far more serious effects on **PHB** and dry weight accumulation, glucose utilisation and protein formation.

This was conducted as a 50-hour experiment, run in a 1l batch reactor, not in a 250ml shake-flask. It demonstrated the ease with which trace elements can be misused (shown on **Figure 10, Chapter 6**). The level chosen to use in the shake-flask experiments, was several times higher than the minimum stipulated. This illustrates how chemicals can be limiting or toxic, if used in too low or high concentrations, respectively. What can be stated, however, was that the use of Cr, Ni and Co, at the level quoted in **Table 3**, was not detrimental. These elements were therefore used in fed-batch and continuous culture experiments, where indeed they may already had become limiting without this extra addition.

The primary assessement of growth was a spectroscopic one, with natural logarithms of absorbance (660nm) plotted against time (hours). The method of obtaining the specific growth rate (μ), was given in **Chapter 4, Section C.1.1.** The specific growth rates obtained, were used to generate values for the **Student's T test.** This statistical technique, is a suitable method to determine significant differences between two sample populations. From **Table 4**, it was relatively self-evident which were the better media, but was this statistically proved? The half chess-board, drawn in **Chapter 4, Section C.4**, described the layout for comparing different media. The technique of obtaining T values was also explained, along with provisional values which were needed. From these,

Table 5

Medium No.	(C5) 1	(C5) 2	(C5) 3	(C5) 4	(C5) 5	TRON 5	(C5) 10	TRON 10	(C5) 11
TRON 11	← 8.817 6dof	← 4.47 12dof	← 3.129 6dof	← 5.108 3dof	← 2.385 3dof	← 4.282 3dof	— 1.669 3dof	— 1.661 2dof	↑ 3.298 2dof
(C5) 11	← 18.722 6dof	← 9.359 12dof	← 10.251 6dof	← 12.297 3dof	← 35.481 3dof	← 18.093 3dof	← 13.785 3dof	← 7.649 2dof	
TRON 10	← 6.792 6dof	← 2.036 12dof	— 0.363 6dof	← 3.695 3dof	— 0.421 3dof	← 2.456 3dof	— 1.087 3dof		
(C5) 10	← 10.872 7dof	← 3.649 13dof	← 1.914 7dof	← 6.502 4dof	— 1.665 4dof	← 5.806 4dof			
TRON 5	← 5.604 7dof	— 0.03 13dof	↑ 2.793 7dof	← 2.677 4dof	↑ 5.979 4dof				
(C5) 5	← 10.57 7dof	← 2.422 10dof	— 1.006 7dof	← 6.299 4dof					
" 4	— 1.645 7dof	↑ 2.469 10dof	↑ 5.326 7dof						
" 3	← 9.645 10dof	← 2.26 13dof							
" 2	← 5.191 13dof								

the following "rules" were observed. If **ten** media were examined, then **nine** columns were required, corresponding to media **one-nine**. There were **nine** rows, starting at the

top with medium **ten**, and going down to medium **two**. In this way, the half chess-board was created, which at a glance was used to compare various media. In this particular instance, there were media 1-5, 10 and 11, and duplication of 5, 10 and 11 for ***Alcaligenes eutrophus*** **TRON**. Thus, a nine by nine half chess-board was required, which was shown on the previous page.

From the half chess-board, scores were assigned to each medium. The arrow pointed to the better medium, when two were compared. Therefore, there were three classes:-

(i) A had a better specific growth rate than B.

(ii) A had a specific growth rate equal to B.

(iii) A had a worse specific growth rate than B.

These three statements were based on a 5% statistical significance level, according to the confines of **Student's T test**. Each medium had a score. For the first five media, only using ***Alcaligenes eutrophus*** C5, these were:-

Medium Score ((i) (ii) (iii), using the points above)

5 (3,1,0)

4 (0,1,3)

3 (3,1,0)

2 (2,0,2)

1 (0,1,3)

From the scores and the half chess-board, it was seen that media 1 and 4 were **equal** to one another, and **worse** than all the other three. Media 3 and 5 were also **equal** to each other, and **better** than all the other three. Medium 2 was **better** than 1 and 4, and **worse** than 3 and 5. There were basically three groups from these two media; media 1 and 4, followed by 2 and finally 3 and 5, in rising order of specific growth rate. This information was used to select a suitable medium, when fed-batch **PHB** storage and continuous culture experiments were examined.

How did the inclusion of a later comparative study of ***Alcaligenes eutrophus*** **C5** and **TRON**, grown on media 5, 10 and 11, change the ranking order? Looking at the half chess-board, the scores became:-

Medium	Score ((i) (ii) (iii), as before)
TRON	11 (6,2,1)
C5	11 (9,0,0)
TRON	10 (4,4,1)
C5	10 (5,3,1)
TRON	5 (2,1,6)
C5	5 (4,3,2)
"	4 (0,1,8)
"	3 (4,2,3)
"	2 (2,1,6)
"	1 (0,1,8)

Once more, **C5** grown on media 1 and 4 had the slowest specific growth rates. Next in order was **C5** grown on medium 2, and **TRON** grown on medium 5. In fact, **C5** grown on medium 5, was better than **TRON** grown on medium 5. Using **C5**, 11 was the best growth medium. This was followed by 10, then 3 and 5, 2 and finally 1 and 4. The **TRON** strain grew fastest on medium 11, once more, but growth on medium 10 was not significantly lower. Growth on medium 5 was, however. In fact, growth of **TRON** was similar to **C5**, on medium 10.

Section A.3. - Discussion of the relevance of each medium, it's specific growth rate (μ) and strain implications.

Medium 1. This was a "general-purpose" medium, which was used to conduct 70-hour fed-batch experiments in the reference. It contained high levels of available nitrogen, phosphorus and carbon. The concentration of potassium was also fairly high, and significantly higher than all other media, with the exception of medium 4. The level of iron (as Fe^{2+}) was also high, some ten-times more so than medium 4, and forty to sixty-times greater than other media. The disparity between media 1 and 4 iron concentrations had no significant effect on the growth rate. The primary problem of using 1g/l $FeSO_4.7H_2O$ was that of precipitation. When media were prepared, the residual pH was about 2. The pH was adjusted using a strong

alkali solution (1-2M KOH or NaOH), to pH 6.8, prior to autoclaving. Medium 1, when initially made up, was clear, but became a very turbid greenish solution, in which the iron rapidly precipitated. Trace element amounts were about 75% of those used in medium 4.

Several possibilities emerge to account for the lowest growth rate, achieved with media 1 and 4. Firstly, high levels of $(NH_4)_2SO_4$ may have been inhibitory. Secondly, the higher levels of potassium may have been toxic, to some extent. Thirdly, iron could have been in excess. Initially, it was thought that the increased concentration of K_2SO_4 might have been responsible. It was shown in **Table 3, Chapter 4, Section B.1,** that media 1 and 4 had 1.5g/l K_2SO_4. This corresponds to a molarity of 0.027M K^+ ions. Media 2, 3 and 5 all have 0.45g/l K_2SO_4, which represents a molarity of 0.008M K^+ ions. However, subsequent analysis of half the shake-flask experiments which were pH controlled, showed that "excessive" potassium was not the problem. This was suprising, in that this was the most comparable similarity of the two media. The ammonium concentrations in both media 1 and 4 were also much higher than all other media, but this was due to the operating regimes of each. Medium 1 was "general-purpose", with large amounts of all available nutrients. Medium 4 was a nutrient limited medium, where phosphate is the initiator for **PHB** accumulation. As a result, $(NH_4)_2SO_4$ had to be increased,

so that a "double-limited" medium did not occur. Thus, ammonium was not the reason for the lowered growth rate. This left only iron. Considering that the levels of iron in medium 1 and 4 were very dissimilar, it was perhaps unusual to find a common effect. The solution to this lay probably in the fact that 100mg/l $FeSO_4.7H_2O$ was too high. Consequently, 1g/l Fe^{2+} would have been even more so. It was likely that as potassium had been excused, 100mg/l of Fe^{2+}, or higher, was too concentrated. This adversely affected growth, producing the drop in growth rate. Medium 4, with 100mg/l $FeSO_4.7H_2O$, actually had a superior growth rate ($\mu = 0.416h^{-1}$) to medium 1 ($\mu = 0.384h^{-1}$), which had 1000mg/l Fe^{2+}. The difference in growth rate was shown not to be significant, although it possibly helped explain the results obtained. Therefore, levels of $FeSO_4.7H_2O$ had to be kept below 100mg/l. This corresponds to a molarity of $3.597*10^{-4}M$ Fe^{2+}. This figure represented nearly thirty-six times the minimum saturating concentration for Fe^{2+} or Fe^{3+}, reported by **Repaske and Repaske (1976[46])**, at $1*10^{-5}M$.

The purpose that medium 1 played in the study of **PHB**, was to grow up **PHB**-free biomass. Initially, it was used by **ICI** as a fed-batch medium, promoting 45g/l cells, with 70% of the cell dry weight as **PHB**, over 70 hours. The long duration suggests that excessive growth has occured first. Results shown in **Chapter 7, Section A.1.(a), (i)-(iv)**, described how such dense cultures were achieved

in less than 48 hours. Therefore, medium 1 was best suited to produce **PHB**-free biomass. It can produce high levels of **PHB**-containing cells, but is an inefficient way to do so. It was, in fact, an early medium, from which others were developed.

Medium 4. Before going onto medium 2, it is probably useful to discuss this one immediately after medium 1, due to the similar growth rates achieved. This medium was "tailor-made" to enable fed-batch storage of **PHB** to occur. The promotion of polymer storage was accomplished by decreasing the level of phosphate. $(NH_4)_2SO_4$ levels were raised, to prevent double limitation of the bacteria. Here, phosphorus was the limiting nutrient, which would run out and cause cessation of the exponential growth phase. **PHB** storage would then occur, during the stationary phase of the cell growth cycle, in the experiment. This particular medium was more suited to studying **PHB** accumulation, than medium 1, in that it had built-in nutrient limitation. **ICI** actually used it in a 250l batch reactor, to get 2.6kg of cells, under fed-batch copolymer production conditions (4-6% **HV** content). It still had too much Fe^{2+}, however, which suggested that more efficient production could be achieved, in a shorter experimental time.

Medium 2. This was again a "tailor-made" medium, which was designed such that nitrogen was the limiting

nutrient. **PHB** would accumulate, during the stationary growth phase, with an excess of carbon (supplied as in a fed-batch system). When the chemical composition of medium 2 was examined, the reason for a greater growth rate than media 1 and 4, was the lowered Fe^{2+} concentration. This equalled $2.698*10^{-5}M$, which was about two and a half times the minimum saturating concentration. Magnesium was present at a lower concentration than medium 4, but higher than medium 1. This indicated that all three concentrations are adequate. The concentration of potassium had been reduced, comparatively from media 1 and 4. This had been discounted as a reason for affecting growth rates.

It was concluded that a concentration of 100 or 1000mg/l $FeSO_4.7H_2O$ was too high. With medium 2, the higher growth rate was attributable to a drop in iron concentration. On the other hand, there was the problem in explaining why medium 2 falls short of media 3 and 5's growth rates. All three media were similar, except for nitrogen and carbon. In addition, medium 2 had <u>half</u> the amount of Fe^{2+}, whilst the quantities of trace elements used in all three were very similar. It is probable, therefore, that instead of media 1 and 4 having <u>too high</u> a concentration of Fe^{2+}, medium 2 had <u>too low</u> a concentration of Fe^{2+}. At $2.698*10^{-5}M$ Fe^{2+} (7.5mg/l $FeSO_4.7H_2O$), this was very slightly above the minimum required by **Repaske and Repaske's (1976[46])** work. In

addition, their work involved the autotrophic cultivation of ***Alcaligenes eutrophus***. In this research, chemoheterotrophic examination of ***Alcaligenes eutrophus*** took place. This would invariably involve quicker growth, which would therefore require an increased amount of Fe^{2+} present in the medium. In media 3 and 5, 15mg/l $FeSO_4.7H_2O$ was present. Thus the difference between the growth rate of medium 2 (μ =0.474h^{-1}) and that of media 3 and 5 (μ =0.523 and 0.539h^{-1}, respectively) was explained. With regard to autotrophic growth, a specific growth rate of 0.347-0.408h^{-1} was achieved. This required at least $1*10^{-5}$M Fe^{2+} to sustain growth. Therefore, 7.5mg/l of $FeSO_4.7H_2O$ ($2.698*10^{-5}$M Fe^{2+}) was probably too low.

Media 3 and 5. Medium 3 was an ICI fed-batch medium, and medium 5 was adapted from it. These are considered as one, with the exception of the provision of extra trace elements in medium 5. This extra inclusion did not affect the growth rate, as both were statistically similar. As the chemical composition was so alike (with the exception of a lower amount of $(NH_4)_2SO_4$ in medium 5), the parity of growth rates was understandable. These two media were statistically better than all previous ones, achieving growth rates of 0.52-0.54h^{-1}. This translates into cell population doubling times of 1 hour 17 and 1 hour 20 minutes, respectively. The concentration of iron used in media 3 and 5 (15mg/l, as $FeSO_4.7H_2O$), encouraged a

faster growth rate than media 1, 2 or 4 iron levels. Due to the similar nature of both media 3 and 5, medium 5 was chosen to experiment further with. The extra trace elements provided a more complete medium, which would be ideal for fed-batch work. The continuous culture medium could have been either, but, apart from one experiment at **Bradford University**, medium 5 was used.

Originally, the intention was to qualify which was the best medium from current available literature. It was not the intention to go into detailed analysis of individual components, as this would be more appropriate to medium development or improvement, which involves considerable work. It is also difficult to draw specific inferences from results, where several components are changed at once. However, in the specific cases of K^+ toxicity or Fe^{2+} precipitation and limitation due to pH control, experimental procedures could be undertaken to accurately determine the solution. Indeed, in **Section A.3.2**, pH control has been investigated, to try to prove whether Fe precipitation was a limitation to growth, in certain circumstances.

Section A.3.1 - Comparative nature of a different strain (*Alcaligenes eutrophus* TRON) and additional media.

Alcaligenes eutrophus **C5** had a growth rate of $0.54h^{-1}$ on medium 5. The production strain, ***Alcaligenes***

eutrophus **TRON**, had a growth rate of $0.475h^{-1}$ on medium 5. With media 10 and 11, the rates were 0.56 and $0.73h^{-1}$ for **C5**, 0.53 and $0.61h^{-1}$ for **TRON**, respectively. Medium 11, used to grow **C5**, produced the fastest growth rate (μ_{max}) of $0.73h^{-1}$. This equates to a cell doubling time of 0.95 hours, or 57 minutes. The specific growth rates (μ) achieved in this research were compared with other strains (used in previous research), are described in **Table 6** on the next page.

The specific growth rates (μ) obtained in this research surpassed all others previously described. It was surprising to note, that the rates for **C5** and **TRON** are higher than those quoted by **ICI** (who ironically supplied both these organisms!). What is also noteworthy, is that **C5** was a faster growing strain than the production strain **TRON**, using the results from this research.

Medium 10 differed significantly from media 1-5, in the lower levels of magnesium used (as $MgSO_4.7H_2O$), and a higher Fe^{2+} concentration. The trace element provision for medium 10 was also slightly more than media 1-5. As was stated earlier, 7.5mg/l $FeSO_4.7H_2O$ was probably too low (medium 2). The level in this medium was 25mg/l, which is probably adequate, without being inhibitory. The concentrations of $(NH_4)_2SO_4$ and H_3PO_4 are also somewhat different, and both were present in potentially limiting

Table 6

Strain	μ (h^{-1})	Reference No.
A. eutrophus) unknown	0.35-0.40	45
A. eutrophus) strain.	0.35-0.46	46
A. eutrophus H/16 ATCC 17699 (DSM 428)	0.42	20
A. eutrophus ATCC 17707	0.31	24
A. eutrophus H/16 S301/C5	0.23	91
A. eutrophus H/16 S301/TRON	≅0.50	91
A. eutrophus H/16 ATCC 17699	0.17-0.46	48
A. latus DSM 1122-24	0.23-0.57	83

DSM = "Deutsche Sammlung von Mikroorganismen", the German type culture collection number.

ATCC = American type culture collection number.

(*Alcaligenes latus* has been listed, due to the inclusion of this organism in the research. This will be described later).

amounts. This provided a medium which was ideally suited for polymer storage, without exponential growth proceeding for too long.

Medium 11 only differs from medium 10 in the amounts of trace elements, magnesium and potassium. Here, the level of Mg^{2+} had been further increased, although the amount present in medium 10 was probably sufficient.

Additionally, the K_2SO_4 was also lower, slightly. The trace elements present differ from medium 10. Copper had been reduced by about half, and manganese was about two-thirds that used in medium 10. The level of zinc was roughly 30% of the amount seen in medium 10. It may have been the case that the trace elements (Cu, Mn and Zn) were slightly inhibitory in medium 10. The inclusion of Na_2SO_4 may also have been important in stimulating the growth rate, over and above the previous 5 media. However, the partial pH control of several shake-flask experiments with NaOH, would probably discount the importance of Na_2SO_4. Medium 10 appears to have been hampered by excessive trace elements (Cu, Mn and Zn). The amounts of $CaCl_2$ (Cr, Ni and Co also) was adequate in both media 10 and 11. Medium 11 has a different, albeit slightly, ammonium and phosphate regime, compared to medium 10. With 11, $(NH_4)_2SO_4$ has been slightly boosted, whilst H_3PO_4 has been reduced. Again, a double limited medium could have resulted, but here phosphate limitation would most likely occur first.

Alcaligenes latus **DSM 1123** was obtained, to examine more comparative work. It was claimed to be much better at producing **PHB**, as polymer storage occurs to about 60% of the cell dry weight, even during normal growth. However, it was difficult to grow, which was confirmed in this research, and a conversation with one of **Chemie Linz's PHB Research team (Hbarak, 1988[85]).** It

grew on salt-free nutrient agar, as it is not tolerant to 5% NaCl, which is present in normal nutrient agar. Liquid cultivation was attempted in shake-flasks and a 2l batch reactor, using medium 5, with sucrose as the carbon source. It did not grow unfortunately! Further examination of the more elusive claims to this bacteria were not possible, within the time-scale of this research. **Table 3, Chapter 4, Section B.1,** listed the actual medium quoted to grow this bacteria, as proposed by **Lafferty and Braunegg (1985[39, 40]).** It was listed merely for reference purposes only, and was not used, even though it was intended to be!

Section A.3.2 - pH control of shake-flasks.

In all, thirty shake-flask experiments were conducted. Of these, fifteen were used to gain the results in this chapter. It was stated in **Chapter 4, Section B.1.1,** that pH control was not done with shake-flasks. Instead, flasks would be pHed before autoclaving, and used as they were. Typically, the residual pH over 8-10 hours, would drop to 6.3-6.1. Of the experiments which were conducted for these results, about half <u>were</u> pH controlled. This was done initially with 0.2M NaOH, and later 0.2M KOH. This did not prolong the exponential growth, nor encourage or discourage the specific growth rate either. If media were run with or without pH control, then no significant effect was

observable (in the results given in **Section A.2**, the shake-flask experiments include the averages for several runs, on the same **medium**). Observation of the standard error of the mean, demonstrates that for medium 2, where four triplicate experiments were run, no significant difference was found between pHed and non-pHed runs). In all cases, exponential growth proceeded for a maximum of about six hours. This was quite short, compared to other work. It seems that lower growth rate populations grew longer. Indeed, this was exemplified by fed-batch work later. For some reason, specific growth rates were significantly lower, using both the same bacteria and media, in a 1 or 2l reactor. Here, the exponential growth phase lasted usually about twelve hours. Considering the procedural difficulties to accurately control pH in shake-flasks (and that pH control did not affect growth, unless the pH fell below 6.3) it was felt unlikely that pH control should be used for further shake-flask study. The use of a buffered medium would avoid any real shifts of pH below 6.3, which would be injurious to the bacteria.

One important consideration of pH control concerns iron precipitation. It was seen how medium 1 had too much Fe^{2+}. This was visualised dramatically when the medium was neutralised, prior to autoclaving. As the pH increased from 2 to 6.8, iron flocculated out of solution. This created a green "fur" at the bottom of the

flask. If the contents were agitated, it became a green, turbid solution. What this warns of, is that any media having less than 25mg/l $FeSO_4.7H_2O$, would be further affected by pH control. Growth rates were lowered if less than 25mg/l Fe^{2+} was used. If very low quantities of iron were used (medium 2), the problem could be exacerbated. In **Section B** of this chapter, the provisional scale-up, to 1 and 2l batch reactors, is covered. This was done to ensure that specific growth rates seen in the shake-flasks, could be adequately reproduced in larger vessels. Experimentally, a 40-60% reduction in growth rate was seen, using batch reactors. Considering the control over various environmental parameters (pH, temperature, dissolved oxygen concentration, etc) is much better in batch reactors, this posed a big question. It was ultimately answered by the fact that pH control was being conducted, using much stronger alkali solutions (0.5M). Therefore, for the media (3 and 5) examined, the iron levels would have been further compromised. A full explanation of this will follow in **Section B.**

Section B.1 - Reactor vessel scale-up, with a reason why shake-flask growth rates were initially not repeatable in batch reactors.

Having obtained a suitable medium, the next stage was to grow up ***Alcaligenes eutrophus*** in a 1l batch reactor. This was necessary, in order to study fed-batch

and continuous culture experiments, which would all involve batch growth initially. It was also a method to ensure the **suitability** of the reactors to be used, **LH 500** series, 1 and 2l vessels, and a **LH 2000** series 16l vessel. Having obtained a high growth rate in shake-flasks was all very well, but it would mean nothing if it could not be reproduced in batch reactors. The set-up of the 500 series (1 and 2l) and the 2000 series (16l) vessels, is seen in the photographs in the **Appendix Section**. The operating conditions were described in **Chapter 4, Section B.1.2 - B.1.4.** The obvious differences between shake-flask cultivation and batch vessel cultivation, were the agitation speed, oxygenation and pH control. Direct oxygenation was possible, due to a sparger in the batch reactor. An oxygen electrode monitored the % dissolved oxygen tension (**DOT**), relative to a 100% air saturated solution. The agitation used a direct, magnetic driven propellor, which was run at 450rpm in the batch reactors. This was chosen initially due to other references works. **Sonnleitner, et al (1979[23])** and **Heinzle and Lafferty (1980[24])**, grew ***Alcaligenes eutrophus*** at 450rpm. It was later seen that a specific **DOT** of 60% was required by ***Alcaligenes eutrophus***, to grow maximally during exponential growth. This work was done at the **University of Bradford**, by **Montgomery (formerly Underhill) and Bitar, (1987[86])**, in a parallel research project on **PHB** storage. This affected only exponential growth, and was adopted later for use in

fed-batch and continuous culture. pH control was initially done with ≡ 0.3M alkali (KOH), but was increased to 0.5M for fed-batch work, and the 16l vessel was adjusted with 3M KOH.

Section B.2 - Results of scale-up.

In the first two experiments, conducted in a 1l batch reactor, and run for just over seven hours, the growth rate was reduced. In both cases, medium 2 was used, which should have given a growth rate of about $0.474h^{-1}$, according to shake-flask data. Here, 0.3M KOH was being used to control pH continually. The growth rate measured was $0.33-0.35h^{-1}$. This meant that a 26-30% reduction in growth rate was encountered. Several reasons for this could have been possible:-

(i) iron precipitation, due to pH control.

(ii) excessive impeller speed, causing shearing of cells

(iii) excessive aeration, leading to oxygen toxicity, or even possibly CO_2 degasification.

(iv) dilution error, as samples were removed, pH control would dilute the residual solution. This would get progressively worse during the experiment.

Due to the results in Sections A.2, A.3 and A.3.2, it

was probable that (i) was the problem. Agitation at 450rpm was not thought likely to be a suitably injurious shear speed. Excessive aeration posed a problem. It was subsequently found that 60% **DOT** was necessary for maximal growth. However, an increase from 60 to 100% **DOT** led to only a 20% drop in growth rate. This, therefore, cannot account wholely for the drop seen in this case. The suggestion that CO_2 could be removed by excessive aeration, and thus limit growth, was made. This arose whilst trying to get a paper published of the early results of this research. Correspondence with the **Editor** of **Biotechnology Letters**, led to this suggestion (**Bu'Lock, 1987**[92]). As a result, an experiment was conducted at **ICI, Billingham**, as part of the **CASE** award industrial session. A 15% CO_2 overpressure was supplied to the aeration gases, to see whether growth was stimulated. This was not the case, however, and CO_2, over and above that found in atmospheric air, was not needed (This was described in **Chapter 7, Section A.1.(a)**).

At various stages of work throughout the research, **DOT** was reduced from 100% to 80%, and adjusted manually (by aeration rate) to compensate for fluctuations. This was later reduced to the final working value of 60% **DOT**. After the **CASE** session at **ICI**, the aeration itself was altered from 1.5vvm (volume of air/ volume of solution/ minute) to 0.3vvm air. This was adequate to supply the cells, and maintain the maximum specific growth rate, in

work done by **ICI**. Finally, an oxygen meter was bought, on which 60% **DOT** could be set, and variation in the **DOT** would trigger the agitator motor speed. When a **DOT** greater than 60% was recorded by the oxygen probe, then the speed would drop, and vice versa. This automatic oxygen concentration control, would be kept within the limits of 250 and 1250rpm, respectively (to maintain 60% DOT). From the results obtained with other experiments (done as fed-batch, or the exponential growth of a continous culture start-up), the reductions in batch reactor growth rate (compared to shake-flask) were as described in **Table 7** below;-

Table 7

Strain	Medium number	Vessel size (l)	Aeration (vvm)	pH control solution (M)	Specific growth rate (μ, h^{-1})	Reduction, (%)
C5	3	1	1.5	0.5M KOH	0.29 - 0.30	45 - 42%
C5	5	1	1.5	0.5M KOH	0.26 - 0.30	52 - 44%
C5	5	16	0.3	3.0M KOH	0.22 - 0.24	59 - 54%
C5	5	2	0.3	0.5M KOH	0.30	45%
TRON	5	16	0.3	3.0M KOH	0.32	32%
(All the above were fed-batch experiments)						
C5	Bradf'd Uni.	1	1.5	0.5M KOH	0.24	no shake-flask data
C5	5	2	0.3	1.0M KOH	0.23 - 0.29	57 - 46%
(The above two were continuous culture experiments)						
C5	2	1	1.5	0.3M KOH	0.33 - 0.35	26 - 30%
C5	5	2	0.3	NIL	0.54	0%
(9-hour maximum batch experiments, to determine effect of scale-up)						

In some of the fed-batch and continuous culture experiments, the exponential growth was severely curtailed. This is especially so in the large, 16l reactors, where 3M KOH was used to control pH. The inclusion of the medium called "Bradford University, continuous culture", was of limited use. This medium, detailed in **Table 3, Chapter 4**, was used for the first continuous culture experiments. No shake-flasks were run using this medium. The growth rate of the start-up of this continuous culture (the exponential phase) was low. pH control was conducted, using 0.5M KOH. The undoubted reason why such a poor growth rate was seen, was the initial iron level. This was just under half the amount used in medium 2, which itself was iron limited. When pH control was started, the level of iron would not have been sufficient, which would account for the low growth rate.

In the last experiment, no pH control was done, and the pH dropped to 6.3, at which point exponential growth ceased. The pH was increased to 6.8, but it was likely that exponential growth was coming to an end anyway, and it did not recover. However, the growth rate was unchanged from the shake-flask experiment. Thus, the reason for non-repeatability (of growth rates, between flask and reactor) was <u>iron precipitation</u>, by pH control. K^+ toxicity was excused in **Sections A.2, A.3 and A.3.2** of this chapter. It may be argued, however, that K^+ toxicity

could be a severe problem in fed-batch culture. This being said, during fed-batch, the greatest amount of alkali would be used during the polymer storage stage. KOH would be used quickest during exponential growth, but would be of limited duration, compared to the storage phase. No details of alkali and cell culture volumes were kept, during longer experiments. A full determination of the effect of K^+ cannot, therefore, be attained. It is possible that K^+ could be toxic in large, fed-batch reactors. It is more likely that the effect of pH control was more subtle, causing iron precipitation, and then slowing growth. The available evidence suggests that iron levels are critical, therefore. This would be especially so for fed-batch and continuous culture experiments.

Section B.3 - Summary conclusions of this chapter.

Several different media were examined, and various suggestions were made:-

(i) ***Alcaligenes eutrophus*** **C5** grew much faster than other previously described strains, with a maximum specific growth rate (μ_{max}) of about $0.73h^{-1}$. This bettered the **ICI** production strain, which had a maximum growth rate of $0.61h^{-1}$. This in itself was somewhat surprising.

(ii) Inclusion of the appropriate levels of chromium, nickel and cobalt, as trace elements, could possibly be

useful to fed-batch work. Levels of limitation and toxicity (too low or too high concentrations, for example) have to be adequately defined, for all nutrients.

(iii) To grow ***Alcaligenes eutrophus*** in fed-batch or continuous culture, to produce biomass free from **PHB** (ie, exponentially grown cells), $FeSO_4.7H_2O$ should be present at a concentration of 25mg/l.

(iv) With these unbuffered media, pH control is probably not necessary to study shake-flask specific growth rates. This is provided the pH does not go below 6.3. In gauging appropriate pHing regimes, especially for fed-batch work, regard must be paid to Fe^{2+} precipitation. This is particularly the case if concentrated alkali is used to supply a large batch reactor. This could deprive the vessel of Fe^{2+}, and stop growth. This problem should also be borne in mind when conducting continuous culture, even when fresh iron will be flowing into the vessel. pH control could still precipitate available iron in these conditions, if insufficient was present initially in the medium composition.

(v) Student's T test provides a suitable statistical analysis, for determining significant differences between two media, supporting the same bacteria.

Further consideration of these points will be given in **Chapter 10, Section 1.** Other work (on fed-batch, continuous culture or multiple-vessel experiments) was used to gauge the validity of these assertions.

Chapter 6 - Single-stage fed-batch experiments.

Initial experiments to study the promotion of **PHB** synthesis, associated analytical techniques and difficulties.

The first objective of the research, as was stated in **Chapter 5, Section A.1**, was to get a "suitable" medium. In this case, "suitable" meant one which allowed rapid cell growth, and could be adapted for use in fed-batch, continuous culture and two-stage vessel experiments.

The results in this chapter were derived from five experiments. These were conducted empirically, in order to gain experience of handling the reactors. Initial determination and problems which occured, were then used to investigate in-depth fed-batch culture.

Section A - Familiarisation experiments, to gain experience of using the reactor vessels.

Two 50-hour fed-batch experiments were done (the results of which are shown in **Figures 7-9** at the end of this chapter), with medium 3 (**Table 3, Chapter 4, Section B.1**). The aeration rate was set to 1.5 vvm, and the air flow rate was manually adjusted to take note of decreasing reactor volume, during sampling. The 1l reactor vessels were filled with about 850ml of medium,

and stirred at 450 rpm. The aeration and agitation rates used, permitted a DOT of about 100%, with respect to air-saturated, uninoculated medium. This decreased to about 70-80%, over the first 8 hours of the experiment, during exponential growth (the DOT was not recorded onto a graph). Ideally, the culture should be operated at a 60% DOT level, with automatic control of either/or stirrer speed and aeration rate. Operating above 60% DOT, up to 100%, gave a maximum drop in specific growth rate of about 20% (**Montgomery (formerly Underhill) and Bitar, 1987**[86]). **PHB** accumulation, however, was unharmed by 100% DOT. By the time that **PHB** formation had taken over as the major metabolic activity, the DOT would already have dropped well below 100%. pH control was achieved with 0.5M KOH. As a result, Fe^{2+} precipitation had probably occurred, leading to a drop in specific growth rate.

The first experiment was of very limited scope. It was used to gain experience of such a long run, using the **LH** 1l vessels. Lessons were learnt from it, which were used and developed in later work. Five experimental parameters were measured. These were absorbance (cell density or turbidity, depicted on **Figure 7**, as experiment 1), glucose, ammonia, dry cell weight (shown on **Figure 8**, experiment 1) and protein concentration (shown on **Figure 9**, experiment 1). The experiment was done without the addition of any more carbon. As such, it was not at all a fed-batch culture, but an "extended-batch" culture. The

reason why excess carbon (as glucose) was not maintained, was to ascertain when, and how much glucose should be added to the culture.

The first indication of growth, and how the reaction was proceeding, was achieved by the absorbance readings. The only difficulty occurred with the overnight range of the experiment. In most cases, this was after the first 12 hours of the experiment. In this particular case, the unrecorded range was from 14.5 - 24 hours. Up until 14 hours (and excluding an initial two-hour lag phase), the specific growth rate (μ) was $0.3h^{-1}$. A study of the plot of absorbance versus time, indicated that exponential growth probably ceased after about 20 hours. The growth rate achieved in this experiment, was 58% of the rate achieved in shake-flasks (using medium 3, **Table 4, Chapter 5, Section A.2**). The point at which exponential growth ceased, was ascertained after consideration of the glucose, ammonia and protein results.

Glucose utilisation proceeded at a rate of 0.75g/l/hour. This was calculated using the data points from 6-24 hours. Even allowing for the fact that the concentration of glucose at 24 hours was 0.17g/l, and then did not markedly decrease throughout the experiment, the rate from 6-24 hours was linear. It was not known, however, whether this concentration of glucose was reached earlier than 24 hours. The results indicate that

glucose would have dropped linearly to about 24 hours, and not much sooner, such as 20 hours. The cells requirement of glucose, at 0.75g/l/hour, with an initial 15g/l medium concentration, should have supported about 20 hours exponential growth. In fact, until 6 hours, glucose utilisation did not occur at this high rate. As a result, the indications are that glucose would have run-out after about 24 hours. Therefore, glucose was probably not preventing exponential growth.

Ammonia (as $(NH_4)_2SO_4$) was used to create conditions of limitation, which themselves would have promoted **PHB** formation. From the experimental results, starting with 4g/l $(NH_4)_2SO_4$, the rate of utilisation was 0.11g/l/hour, from 8 - 30 hours. Experimentally, it appeared that there was less $(NH_4)_2SO_4$ than originally planned, in the initial medium composition. It appeared, as was the case with glucose, that as the cells grew in the first 6 hours, the uptake was not significant, due to low cell densities. However, the rate of utilisation of ammonia was linear from 8 - 30 hours. This would still be the case if the initial concentration of 4g/l (at 0 hours) had been included. If 0.11g/l $(NH_4)_2SO_4$ was used every hour, then the medium should have supported approximately 36 hours of growth. In addition, where the level apparently did not drop for the first 8 hours, this probably would have allowed growth until about 44 hours. From the results, it was seen that protein increased

significantly between 9 - 27 hours, at a rate of 0.12g/l/hour. The situation was, therefore, that glucose would most likely have run out after about 24 hours, whilst ammonia was still present. Growth probably proceeded until 20 hours. The measurable rise in dry weight occured from 8 - 27 hours. This decreased afterwards, from a maximum of 6.74 down to 2.94g/l. In this experiment, instead of having an adequate carbon source, and potentially limiting nitrogen conditions, the opposite was the case. Due to the results, it appears that growth proceeded for about 20 - 24 hours, when glucose became limiting. Consequently, three hours later, the cell density reached it's maximum, and decreased steadily thereafter. It was highly unlikely that **PHB** was produced to any significant extent. Indeed, the maximum cell dry weight contained about 35% protein. Under normal circumstances, residual biomass accounts for 20 - 40% of the cell weight.

The next experiment had to include several fundamental changes. It was shown that glucose was utilised at 0.75g/l/hour. This would lead to carbon limitation after 20 hours, with an initial glucose concentration of 15g/l. Consequently, the glucose was increased to 20g/l, which would allow for over 24 hours utilisation. This made for better experimental management, without the need for continual hourly samples to be taken. Whilst carbon was increased, conversely

nitrogen was decreased. In the previous experiment, it was seen how exponential growth took place for virtually 20 hours. Ideally, at this stage, ammonium would be limiting already, and promoting **PHB** production. There are advantages in getting very dense **PHB**-free cell cultures, in that the amount of **PHB** formed will be greater. This is offset by the fact that longer experiments are required. This is especially the case if an industrial process to make **PHB** was ultimately required. A balance has to be struck, between the extra cost of longer experiments, and the extra value of **PHB** gained by increased culture. Consequently, $(NH_4)_2SO_4$ was reduced from 4 to 2g/l. This would lead to nitrogen limitation occurring first. The final alteration was the provision of additional glucose. Because very small additions of glucose (in terms of volume, in order not to dilute the culture) were required, 5g/20ml solutions were used. The concentrated solution was sterilised in 20ml sample bottles, and injected into the culture using a sterile hypodermic syringe. This was, therefore, the first **fed-batch** experiment, under the definition given in **Chapter 3, Section 3.3.**

The results of the changes in the second experiment, led to the following results (depicted in **Figures 7-9**, experiment 2). Again, using the absorbance of the culture to gauge the turbidity or cell density, exponential growth occurred for at least 14 hours. By

looking at the graph (**Figure 7**, in particular the difference between the values at 14 and 24 hours), it was apparent that the exponential growth ceased shortly after 14 hours. The specific growth rate (μ) was $0.29h^{-1}$, which was 55% of that achieved in shake-flask culture, using medium 3 (**Table 4, Chapter 5, Section A.2**). The reason for this decrease was almost certainly a result of pH control. This was probably not, however, a case of K^+ toxicity, but more than likely a problem of Fe^{2+} solubility.

Fe^{2+} is necessary in the formation of the various cytochrome molecules, which are all derived from haem molecules. These cytochromes, in conjunction with Fe^{2+} and Fe^{3+}, are used in the electron transport chain. This is the major source of energy, at the end of the **Kreb's tricarboxylic acid cycle**. ATP is generated, and NADH reoxidation also takes place. ATP is one of the most valuable and potent sources of energy available to the cell.

What seemed to be occuring, was that in medium 3, the level of Fe^{2+} (as $FeSO_4.7H_2O$) was reduced below the minimum saturating concentration. This probably deprived the cell of enough cytochrome, and decreased the rate at which the electron transport chain proceeded. This was described in **Chapter 5, Section B.2.**

The increase in the original glucose concentration should have allowed for at least 24 hours growth. It was planned to add glucose at 25 and 35 hours, at the beginning and end of the second day. Unfortunately, glucose had already run-out after 24 hours (**Figure 8**, experiment 2). From the results, glucose utilisation had increased from 0.75 to 0.83g/l/hour. The increased utilisation meant that glucose would barely last 24 hours. However, judging by the results of absorbance and cell weight, glucose probably did not run-out until just before 24 hours. The provision of extra glucose at 25 and 35 hours (when the volume of culture was 600 and 500ml, respectively, at these times), resulted in glucose concentrations of 8.3 and 10g/l. Consequently, the experiment did not suffer from any apparent limitation. The addition of extra glucose allowed for the probable synthesis of **PHB**. During the stationary phase, glucose utilisation remained between 0.8-0.6g/l/hour.

The actual mechanism by which **PHB** was to be encouraged, was achieved by lowering the initial nitrogen concentration. With the previous rate of $(NH_4)_2SO_4$ utilisation (0.11g/l/hour), and 2g/l present in the medium, nitrogen should have become limiting after about 18 hours. In fact, the initial concentration of NH_4^+ was apparently only 1.18g/l (**Figure 8**). The rate of utilisation was also lowered, to 0.08g/l/hour. In actual fact, nitrogen should only have lasted between 14 and 15

hours. After 14 hours, it had expired, which would then have stopped exponential growth. This was in accordance with the findings of the absorbance readings. The resultant protein (**Figure 9**, experiment 2), was formed at 0.05g/l/hour. This would again suggest that the level of initial nitrogen was too low. Protein did not increase in concentration, significantly, from 14 hours until completion of the experiment. After 48.5 hours, the cell dry weight reached a concentration of 9.52g/l (**Figure 8**), of which 5% was protein. If it is assumed that 20-40% of the cellular material is "residual biomass" (**PHB** and protein free), then the probable level of **PHB** was 55 to 75%. In the final 1.5 hours, the dry weight dropped, corresponding to secondary limitation of another mineral (other than carbon, as glucose was still present at shutdown) or cell lysis. The precise determination of percentage **PHB** was not possible at this stage of the research. Assay techniques for **PHB** determination were obtained and used in later experiments.

These two familiarisation experiments can be summarised into the data table (**Table 8**) shown on the next page.

With both of these experiments finished, problems, solutions and suitable developments emerged. The primary problem was sampling. It would be ideal to do more sampling, with far less lengthy gaps, alleviating the

Table 8

Fed-batch experiment no.	1	2
Duration (hours)	50.0	50.0
Medium (Table 3, Chapter 4)	3	3
Initial [glucose] (g/l)	15.0	20.0
Initial $(NH_4)^+$ (g/l)	4.0	2.0
Carbon feed (y/n)	no	yes, 25 and 35 hours
Specific growth rate (μ, h^{-1})	0.30	0.29
Exponential growth duration (h)	20.0	14.0
Glucose utilisation: (a) growth (g/l/h)	0.75	0.83
(b) polymer storage	0.0	0.8 - 0.6
Nitrogen utilisation (g/l/h)	0.11	0.08
Protein formation (g/l/h)	0.12	0.05
Probable [PHB] level (%)	25 (-40)	55 (-75)
Maximum cell density (g/l)	6.74 (27h) 2.94 (50h)	9.52 (48.5h) 9.20 (50h)
Comment:-	C-limited!	N-limited, reasonable run

overnight ten-hour interval. Two physical considerations of sampling were realised: there was the need to prevent the reactor from being emptied, and to reduce dilution by alkali addition during pH control. Details of the procedures taken to account for these were given in **Chapter 4, Section B.1.3.**

Using the scheme proposed in the **Chapter 4, Section C.3**, graphs 1-3 were completed. The next experiments would require a method of determining **PHB** accurately, and total cell counts would have to be done. This would again exacerbate the sampling "problem".

Section B - Fed-batch experimentation, with PHB analysis.

Medium 5 was used for the later experiments. The main difference from medium 3 was the inclusion of additional trace elements, Cr, Ni and Co (according to the reference of **Repaske and Repaske (1976[46])**). Whilst a significant change in growth rate was not seen for medium 5 (over medium 3), the inclusion was thought prudent. In addition, considering the grades of chemicals used (mainly **BDH AnalaR** ones), the level of available Cr, Ni and Co was, to all intents and purposes, nil.

The first experiment was run using medium 5, but a mistake in medium preparation was made. Instead of using 1ml/l stock trace elements (Cr, Ni and Co), 10ml/l was

incorrectly added. The stock trace metal element solution, was formulated to give more than the minimum concentrations needed. The use of ten-times too strong medium had a somewhat disastrous effect on the experiment. As a result, the experiment only lasted 35 hours. The absorbance (cell density) readings were lower, which led to severe depression of the growth rate (**Figure 10**, experiment 1). The specific growth rate (μ) was only $0.22h^{-1}$, about 41% of the shake-flask results, using medium 5. Exponential growth occured during 2 - 8.5 hours, when glucose utilisation was very low. Indeed, after growth stopped, the glucose concentration actually built-up. This was exaggerated by glucose addition at 11, 24 and 32 hours. Ammonia was used at a very low rate, 0.02g/l/h (**Figure 11**), and protein increased at 0.019g/l/h (**Figure 12**) from 0 - 35 hours. Nitrogen was actually still present at shutdown (**Figure 11**), when the cell dry weight was 3.04g/l (**Figure 11**), and protein was 0.54g/l (18%). This experiment served to illustrate how chemicals can limit or inhibit growth, in inappropriate concentration (too low or too high, respectively).

A repeat of the experiment was performed, with Co, Ni and Cr at the correct level. This time the indications were better, as absorbance readings climbed faster. The specific growth rate (μ) (as determined by the absorbance, **Figure 10**, experiment 2) was $0.26h^{-1}$. This was 48% of the shake-flask results. Exponential growth

proceeded, after a lag phase of two hours, until 14 hours. The results suggest that this probably continued until 16 - 17 hours. For the first time, total cell count measurements were included, which gave a much higher growth rate. The plot of ln (log_e) total cells/ml versus time (**Figure 13** experiment 2), gave a specific growth rate (μ) of $0.42h^{-1}$. The reason for the discrepancy was unclear, but probably arose out of counting and dilution errors. At the end of the experimental growth phase (13 - 15 hours), the cell density had reached $1.7*10^{10}$ cells/ml. After 37 hours, this reached $4.28*10^{10}$ cells/ml, and dropped back to $3.49*10^{10}$ cells/ml after 48 hours. The 18.5% drop in cell number was indicative of a certain amount of cell lysis. As cells accumulated more and more polymer, they would gradually lose viability, die and lyse. Intact cells would still continue to store more **PHB**, so the overall dry weight would increase (as liberated **PHB** granules would persist in the medium). The increase, however, would be less pronounced than before lysis took place.

During exponential growth, glucose utilisation proceeded at a rate of 0.8g/l/hour (**Figure 11**, experiment 2). Glucose additions occured after 13.3, 24 and 33 hours, when 7.5g of glucose (in 20ml of sterile deionised water) was injected. The culture volume was about 500ml at both of these times, so the increase in glucose was 15g/l. The glucose utilisation decreased to 0.6 -

0.5g/l/hour, during polymer storage. The increase in **PHB** was indicated by the cell dry weight reading, which was noticeable from 8 hours into the experiment. The dry weight increased until 37 hours, and then it slowed until shutdown. The rate of weight increase was very stable, at 0.37g/l/hour. The final cell concentration during this fast increase was 12.2g/l, which rose to only 12.4g/l at the end (**Figure 11**, experiment 2).

The level of available nitrogen (as NH_4^+) was 3g/l. Nitrogen utilisation took place at a rate of 0.21g/l/hour (**Figure 11**, experiment 2), whilst protein accumulated at 0.05g/l/hour (**Figure 12**, experiment 2). This was distinct from the other experiments, where protein accumulation took place at a slightly lower rate than nitrogen utilisation. The main observable difference was that nitrogen utilisation was higher. If the additional trace elements are considered, then this was a logical situation. Cobalt, particularly, is required as a cofactor for enzymic activity, during the production of several very important chemicals. This includes, for example, the synthesis of **adenine** and **cobalamin (vitamin B12)**. Adenine is necessary for the production of **Coenzyme A, ATP, NADH**, etc. The assimilation of ammonia, in the formation of proteins, is affected by cobalt. A specific example can be seen in the **lysine** formation pathway. The conversion of **N-succinyl-LL-2,6-diaminopimelate** into **LL-diaminopimelate**, is facilitated by the action of the

enzyme **Succinyl diaminopimelate deacylase.** Water and cobalt are required by the reaction. During rapid growth, lysine formation would require cobalt. This would then speed-up the assimilation of nitrogen, and increase the specific growth rate (μ). In this experiment, nitrogen utilisation was enhanced.

PHB analysis was done with the use of a gas chromatograph. Polymer started to accumulate appreciably from 9.5 hours (**Figure 14**, experiment 2), even though nitrogen was still present (as NH_4^+) at 1g/l. The **PHB**, at a concentration of 0.11g/l, was 6.5% of the cell dry weight. This fits in with the 10% limit of **PHB** storage during exponential growth. At shutdown, the **PHB** concentration had risen to 7.87g/l, which was 63.5% of the cell dry weight. With protein accounting for 11% of the **PHB**-free biomass, the "residual biomass" was 25.5%. In addition to compiling all of this data, several other facts were obtained.

Section C - Additional results.

Yet more results were derived from the earlier ones. One particular parameter used for derivation was cell counting. The average weight of a cell could be calculated, which would reveal more of the physiology of **PHB** storage. A "standard" number of cells was chosen, so as to get an average weight versus time. Since the

majority of cell counts were around $1*10^{10}$ cells/ml, this was adopted as the "standard". So, for example, if after 4 hours the cell density was $1.267*10^{9}$ cells/ml, which had a dry weight of 0.3g/l, then the weight would have to be multiplied by a factor. The factor would be the amount necessary to get the cell count to $1*10^{10}$ cells/ml. This can be calculated as follows:-

$$\frac{\text{"standard" cell count } (1*10^{10} \text{ cells/ml})}{\text{actual cell count}}$$

If the actual count is less than $1*10^{10}$, then the multiplication factor is more than 1, and vice versa. In the case quoted, the factor required to bring $1.267*10^{9}$ to $1*10^{10}$ is 7.89. Therefore, at 4 hours, the dry weight of a standard $1*10^{10}$ cells/ml, would be 2.366g/l. After 27 hours, the cell density was $2.47*10^{10}$ cells/ml. The weight of these cells was 7.75g/l. Thus, the average weight of $1*10^{10}$ cells/ml, after 27 hours, was 3.138g/l. The results of the entire experiment give some idea of the physiology of the cells, during exponential growth and polymer storage. During exponential growth, the cells remain largely of a uniform weight, with an equally uniform **PHB** weight.

This was the view of **Herbert (1961[93])**. During exponential growth, the cells had an average weight of 0.18-0.25pg/cell (pg = picogram, or $1*10^{-12}$g). This was

in agreement with **Herbert's** figures, for bacteria of a similar shape and size. The average cell weight is higher in exponentially growing cells. Greater quantities of protein, DNA, RNA, etc, are formed as cells divide. Stationary or lag phase cells are of lower average cell weight. This assumes, however, in the case of the stationary phase, that storage products are not accumulated. This is invalidated when examining ***Alcaligenes eutrophus***, and other reserve polymer storing bacteria, of course. In this experiment, however, it was noticed that the average cell weight and **PHB** concentration/cell, increased during the storage phase only (**Figure 15**, experiment 2). Prior to **PHB** accumulation, results were lacking. It was seen, however, that the increase in cell dry weight was 0.07-0.09g/l per $1*10^{10}$ cells/ml per hour. The increase in **PHB** was $0.11g/l/1*10^{10}$ cells/ml/hour. It is therefore the case, that **PHB** accumulation accounted for all weight increases, during the stationary (storage) phase.

Another important derivation of the results would ascertain the maximum rate of **PHB** storage, and at which cell content of **PHB** (%). The results of the **PHB** accumulation versus time could be used for this derivation. The rate of accumulation for progressive samples was calculated. These accumulation rates were plotted against the actual %**PHB** content of the cells (**Figure 16**, experiment 2). A maximum **PHB** accumulation

rate of 0.316g/l/h was achieved. This took place at a **PHB** content of 60%. For cells containing less than 60% **PHB**, the rate of polymer accumulation would increase steadily. Above 60%, the rate dropped sharply. What does this actually prove, however? The rate and levels achieved, differed from **Sonnleitner, et al (1979[23])**, who used ***Alcaligenes eutrophus* H/16 ATCC 17699.** Their organism accumulated **PHB** to 0.6g/l/hour, but only to a **PHB** concentration of 30% of the cell dry weight. Obviously, ***Alcaligenes eutrophus* H/16 S301/C5** was more tolerant of **PHB** accumulation. Conversely, it was less efficient at storing the polymer.

Subsequent results confirmed these findings, and filled in some "holes" in the data. The specific growth rate (μ) in the last single-stage fed-batch experiment was $0.3h^{-1}$ (from **Figure 10**, experiment 3). Exponential growth took place from 1-13.25 hours, and would have ceased within a couple of hours of this point. The growth rate was 56% of the shake-flask result. Once again, the total cell count growth rate was different, this time lower at $0.26h^{-1}$ (**Figure 13**, experiment 3). This was, however, a better set of results, which were not too distinctly different. Carbon assimilation occured at 0.55g/l/hour (**Figure 11**, experiment 3), and nitrogen was used at 0.12g/l/hour (**Figure 11**). The dry cell weight increased in a similar manner to the previous experiment. The rate of increase was 0.32g/l/hour (**Figure 11**), which

occured from the late exponential stage until 40 hours. After this time period, the level of glucose was very low, which halted any significant further increase in cell weight. The **PHB** results were somewhat erratic (**Figure 14**), which was indicative of growing preparatory difficulties. The result of rate of **PHB** accumulation versus %**PHB** content of the cells gave a similar result: 0.3g/l/hour storage, up to 55-60% **PHB** content (**Figure 16**). The maximal **PHB** concentration was 6.462g/l, which corresponded to 59% of the cell dry weight, after 42 hours. Both the **PHB** results and the **PHB** accumulation versus % storage, have to be taken in the context of the preparatory difficulties. Only with later techniques for **PHB** analysis was the full result known. In the residual eight hours of the experiment, the **PHB** concentration decreased, indicating the lack of glucose. The average weight of the cells during exponential growth was 0.29-0.35pg/cell (**Figure 15**), again within the range reported by **Herbert (1961[93])**.

These experiments, conducted with medium 5, were all operated at an aeration rate of 1.5vvm. The impeller speed was 450rpm, and pH was controlled using 0.5M KOH.

The results of the last 3 assayed fed-batch experiments are summarised into **Table 9**, shown on the next page.

Table 9.

Fed-batch experiment number	3	4	5
Duration (hours)	35.0	50.0	50.0
Medium number	5*	5	5
Initial glucose (g/l)	20.0	20.0	20.0
Initial $(NH_4)^+$ (g/l)	2.0	3.0	3.0
Carbon feed (y/n)	yes 13, 24 and 32 h.	yes 13, 24 and 33 h.	yes 13, 24 and 34h.
Specific growth rate (μ, h^{-1})			
(a) absorbance	0.22	0.26	0.30
(b) cell counts	ND	(0.42)	0.26
Exponential growth duration (hours)	8.5	16 - 17	13 - 15
Glucose utilisation (g/l/h)			
(a) growth	ND	0.8	0.55
(b) polymer storage	ND	0.6 - 0.5	0.55
Nitrogen utilisation (g/l/h)	0.02	0.21	0.12
Protein formation (g/l/h)	0.019	0.05	0.07
Cell density			
(a) g/l	3.04 (35h)	12.4 (50h)	11.0 - 11.7 (42 & 50h)
(b) cells/ml ($*10^{10}$)	ND	3.505	2.169
Final protein concentration (g/l)	0.54	1.34 (48h)	1.25 (50h)
PHB (g/l)	ND	7.87 (50h)	6.46 - 5.43 (42 - 48h)
%PHB accumulation	ND	63.5 (50h)	59 - 46 (42 - 48h)
Maximum PHB formation rate (g/l/h)	ND	0.316	0.281
Maximum PHB content of cells, above which PHB formation drops	ND	55 - 60%	55 - 60%

* with ten-times too much trace metal (Co, Cr and Ni).

All of these single-stage fed-batch experiments, were done to gain experience in making **PHB**, on a very small scale. The experimental design was developed empirically, which is why the results have been recounted one at a time. Further fed-batch data was gained in the work using 2 and 16l multiple-vessel systems. This is described in **Chapter 9.**

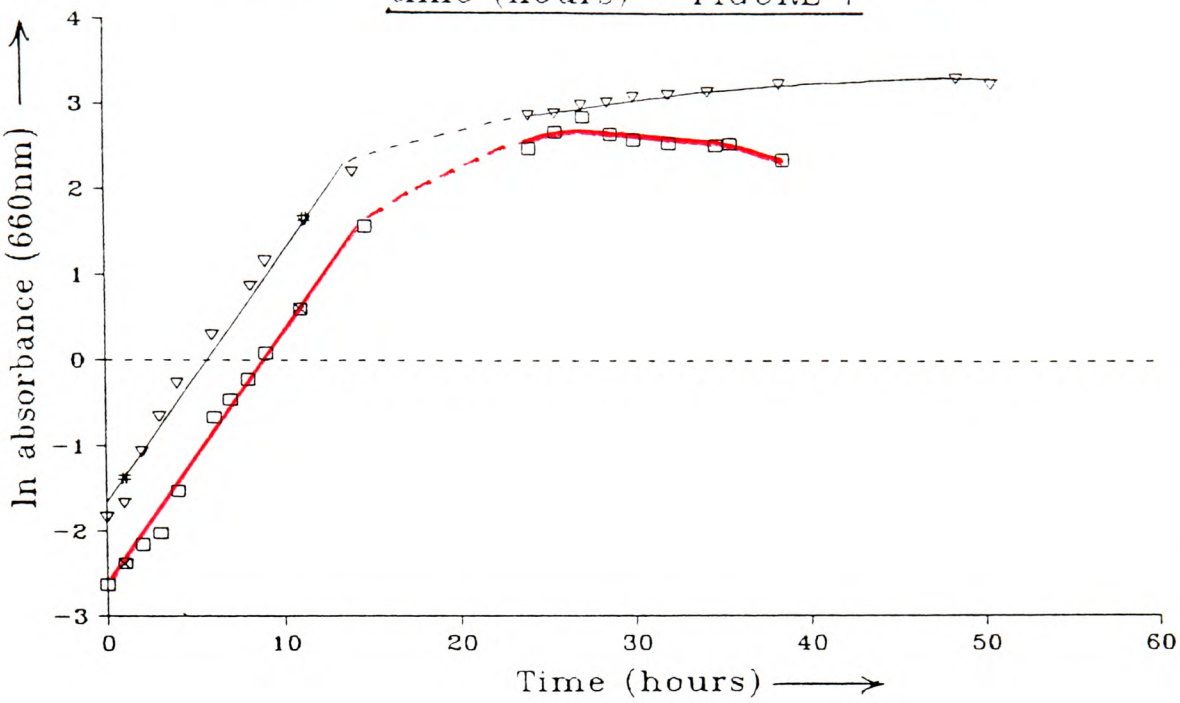
Section D - Experimental difficulties and possible solutions.

One of the major problems of these early experiments was the reactor vessel size. A 1l vessel usually allowed only 400ml of residual cell culture. This meant any recovery of **PHB** would have to be efficient to get the small quantity of polymer present. The polymer was recovered in order to analyse purity, quality and extraction techniques. The immediate solution to too little capacity, was answered by converting the LH 500 series 1l vessels to 2l. This was done by merely replacing the glass reactor vessel, and using longer probes etc. A 2l reactor was used with medium 5 (using the conventional operating conditions), run in a fed-batch mode. As a result, a 50-hour run yielded 16g of cells, containing about 60% **PHB**. This provided enough **PHB** to recover and use for analysis, at **ICI, Billingham.** After receiving the "recipe" for media 10 and 11 from **ICI,** a 2l reactor of each was started, again over

50-hours and unassayed. Unfortunately, less **PHB** was recovered than was originally hoped for. The results of **Chapter 9** provide further data on fed-batch culture.

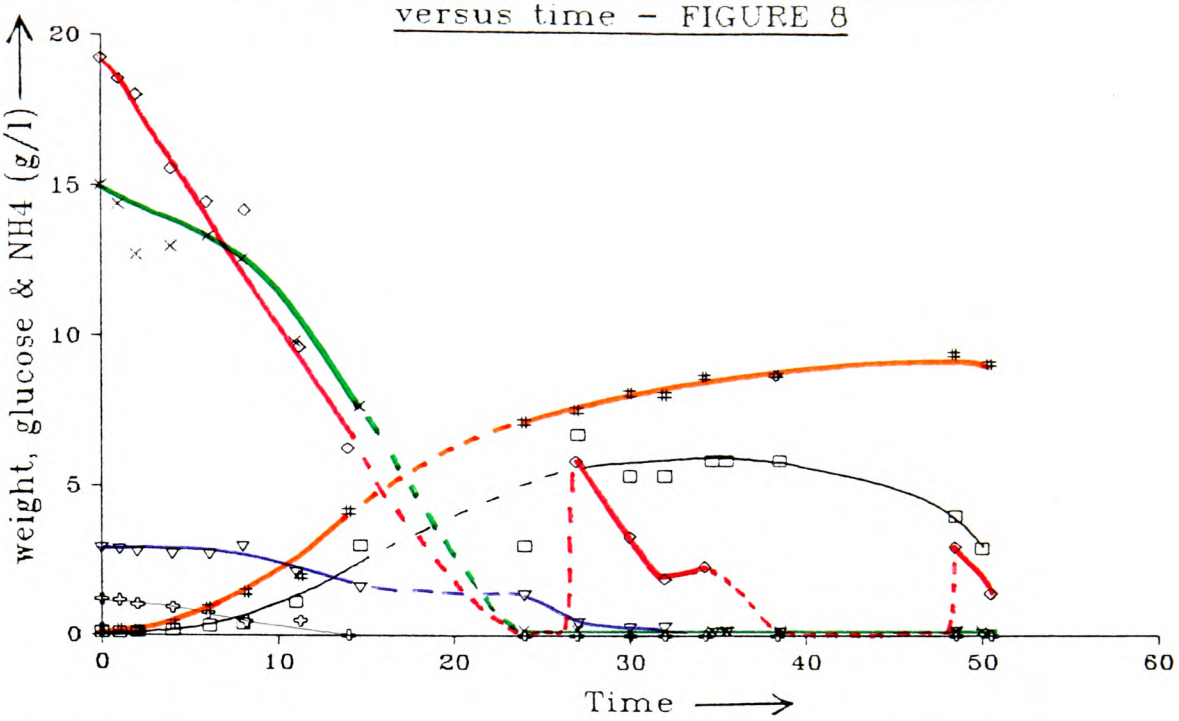
There were several benefits of using vessels larger than 1l. For fed-batch work, sampling would not excessively deplete the vessel of culture volume. The increased volume could also allow for more details to be ascertained, from other analytical techniques. In addition, the actual procedure of automating fed-batch was possible. Peristaltic pumps could be used to accurately pump in a small quantity of concentrated glucose, over a period of time. Providing Fe^{2+} levels were regulated properly, pH control could use stronger alkali solutions. This would cut down on the volume used, which would lessen dilution of the culture. Whilst this chapters work has been fairly restricted, ideas and schemes were rationalised. From this, multiple-vessel fed-batch or continuous culture would be better understood, albeit with further data. When the problems encountered in this work were taken into account, they provided a useful introduction to the remaining work.

ln absorbance (660nm) versus time (hours) – FIGURE 7



Time (hours)		ln absorbance (660nm)		Best fit	
.000	.000	-2.631	-1.845		
1.000	1.000	-2.386	-1.671	-2.384	-1.374
2.000	2.000	-2.163	-1.070		
3.000	3.000	-2.025	-.660		
4.000	4.000	-1.523	-.274		
6.080	6.000	-.667	.287		
7.000	8.170	-.462	.862		
8.000	9.000	-.223	1.155		
9.000	11.250	.081	1.636		1.664
11.000	14.000	.597	2.194	.610	
14.670	24.000	1.569	2.868		
24.000	25.500	2.485	2.890		
25.500	27.000	2.674	2.989		
27.080	28.500	2.845	3.016		
28.670	30.000	2.649	3.085		
30.000	32.000	2.586	3.109		
32.000	34.250	2.549	3.144		
34.670	38.333	2.518	3.232		
35.500	48.500	2.539	3.313		
38.500	50.500	2.355	3.254		
1.	2.	1.	2.	× 1.	# 2.

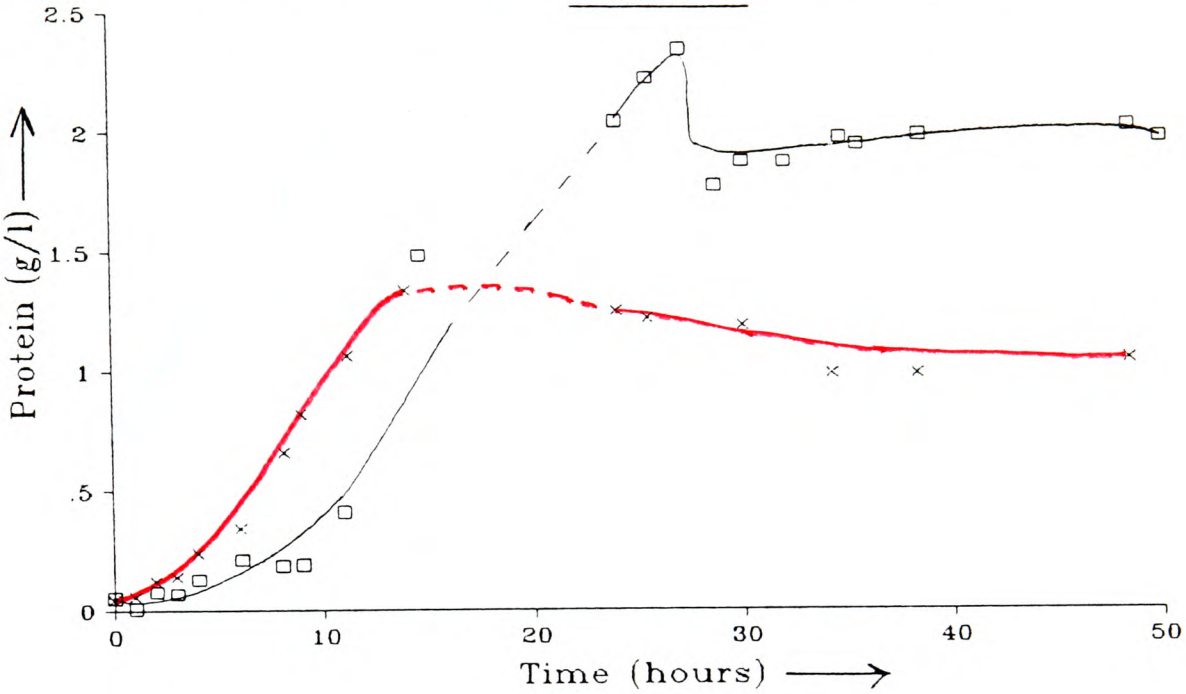
Dry weight, glucose & NH4
versus time - FIGURE 8



Time (hours)		Dry cell weight (g/l)	Glucose	NH4	Dry cell weight (g/l)	Glucose	NH4
.000	.000	.095	15.000	2.880	.175	19.220	1.177
1.000	1.000	.070	14.370	2.840	.180	18.540	1.158
2.000	2.000	.115	12.700	2.760	.150	18.000	1.024
4.000	4.000	.170	12.970	2.690	.400	15.550	.919
6.080	6.000	.310	13.330	2.690	.900	14.460	.757
8.000	8.170	.370	12.560	2.960	1.450	14.190	.455
11.000	11.250	1.100	9.840	2.090	1.980	9.650	.484
14.670	14.000	3.000	7.670	1.600	4.160	6.260	.000
24.000	24.000	3.000	.170	1.330	7.160	.000	.000
27.080	27.000	6.740	.170	.410	7.560	5.850	.000
30.000	30.000	5.360	.170	.250	8.160	3.320	.000
32.000	32.000	5.360	.160	.290	8.120	1.910	.000
34.670	34.250	5.900	.160	.090	8.720	2.320	.000
35.500	38.333	5.900	.160	.130	8.800	8.760	.000
38.500	48.500	5.900	.150	.100	9.520	2.990	.000
48.500	50.500	4.060	.150	.140	9.200	1.420	.000
50.000		2.940	.150	.050			
1	2	1			2		

Protein (g/l) vs Time (hours)

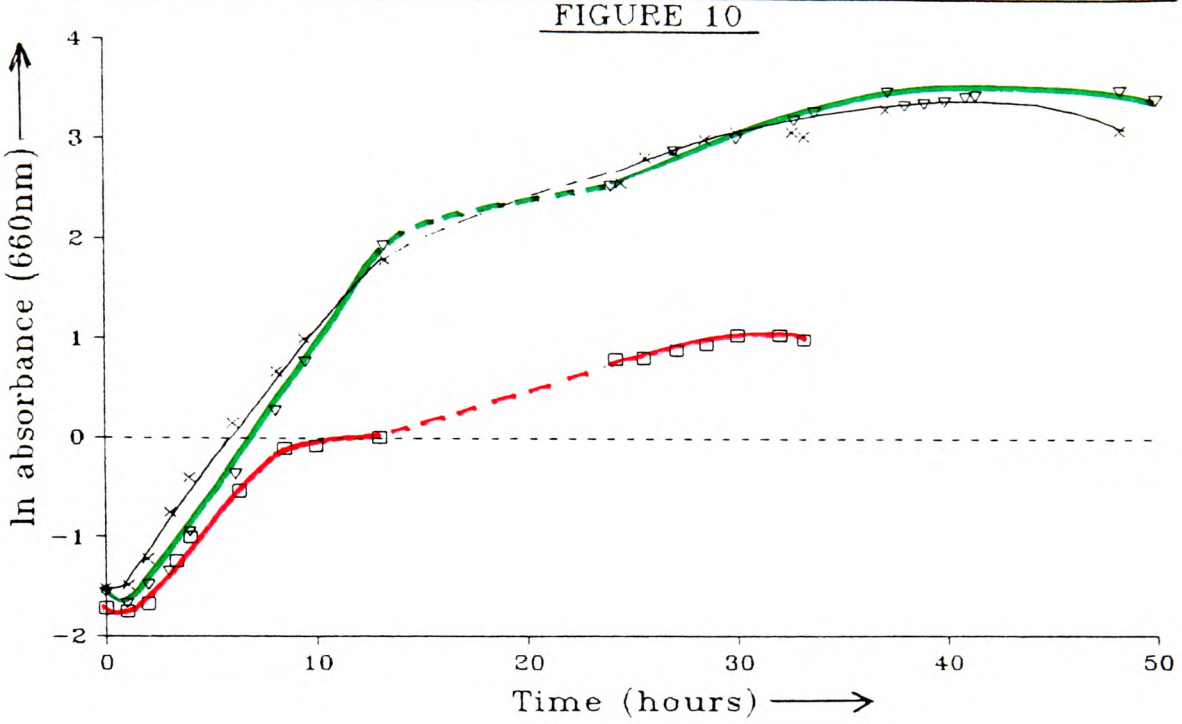
FIGURE 9



Time (hours)		Protein (g/l)	
.000	.000	.052	.044
1.000	1.000	.007	.059
2.000	2.000	.078	.120
3.000	3.000	.068	.140
4.000	4.000	.129	.242
6.080	6.080	.211	.340
8.000	8.170	.185	.660
9.000	9.000	.191	.823
11.000	11.250	.410	1.067
14.670	14.000	1.488	1.340
24.000	24.000	2.050	1.251
25.500	25.500	2.230	1.220
27.080	30.000	2.352	1.190
28.670	34.250	1.780	.986
30.000	38.333	1.882	.986
32.000	48.500	1.878	1.047
34.670		1.980	
35.500		1.953	
38.500		1.994	
48.500		2.031	
50.000		1.980	
1	2	1	2

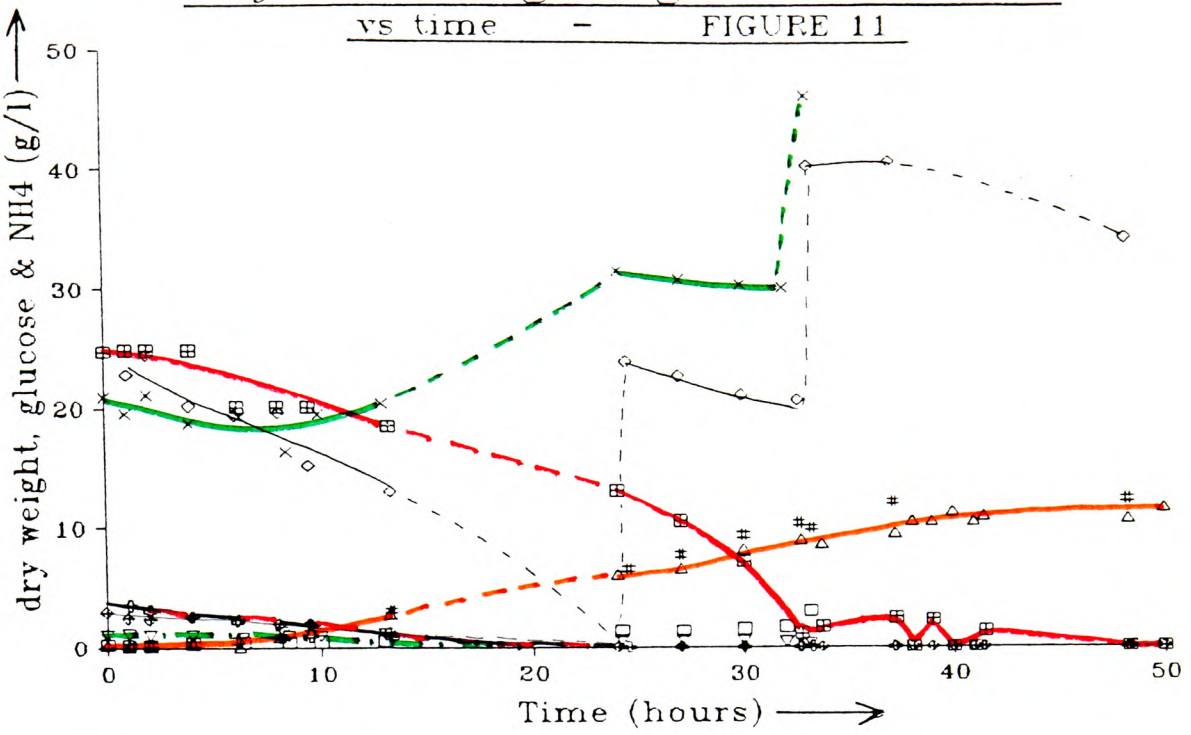
ln absorbance (660nm) vs time (hours)

FIGURE 10



Time (hours)			Absorbance (660nm)		
.000	.000	.000	-1.720	-1.519	-1.570
1.000	1.080	1.000	-1.754	-1.486	-1.677
2.000	2.000	2.000	-1.677	-1.226	-1.487
3.333	3.080	3.000	-1.248	-.749	-1.343
4.000	4.000	4.000	-1.004	-.405	-.949
6.333	6.080	6.170	-.532	.149	-.367
8.500	8.170	8.080	-.104	.664	.266
10.000	9.500	9.500	-.085	.999	.763
13.000	13.333	13.250	.003	1.791	1.929
24.170	24.500	24.000	.798	2.569	2.534
25.500	25.670	27.000	.805	2.820	2.882
27.080	27.000	30.000	.889	2.887	3.014
28.500	28.500	32.750	.945	3.009	3.204
30.000	30.000	33.750	1.040	3.084	3.281
32.000	32.670	37.250	1.043	3.083	3.489
33.170	33.250	38.080	1.002	3.036	3.359
	37.170	39.000		3.316	3.380
	48.333	40.000		3.111	3.403
		41.000			3.448
		41.500			3.458
		48.333			3.511
		50.000			3.418
1	2	3	1	2	3

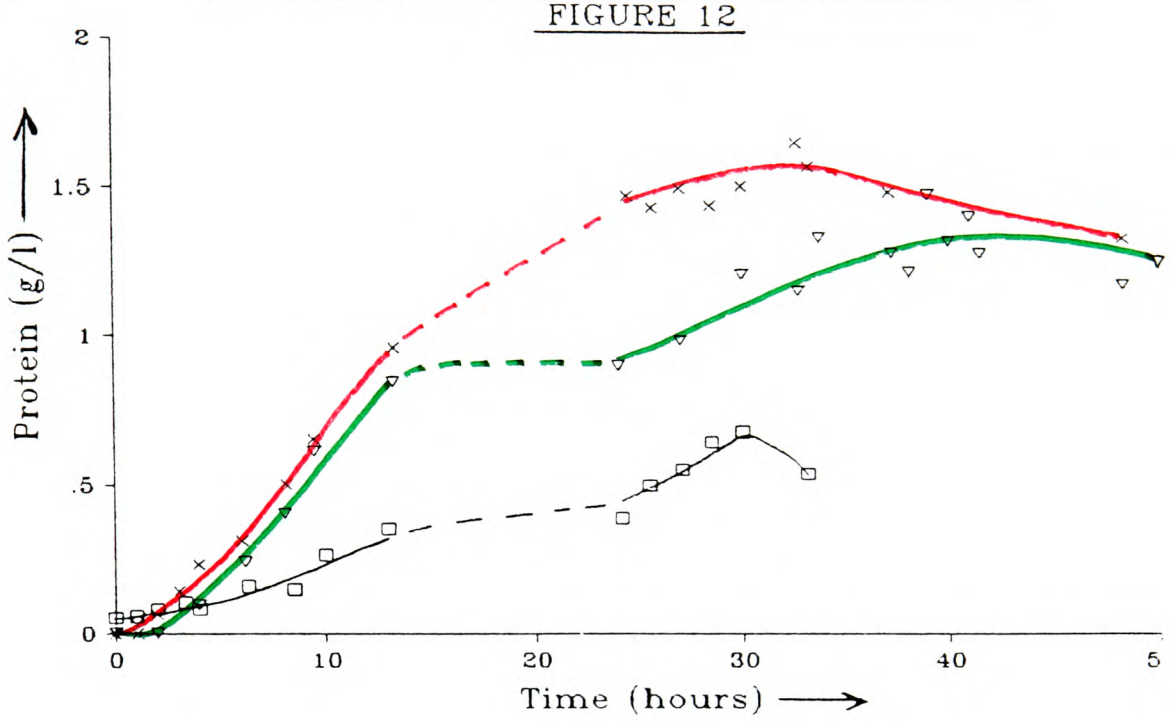
Dry cell weight, glucose & NH4 vs time - FIGURE 11



Time		(hours)	Dry wt	gluc	NH4	Dry wt	gluc	NH4	Dry wt	gluc	NH4
.000	1.080	.000	.213	20.954	1.080	.200	22.802	3.534	.200	24.737	2.964
1.000	2.000	1.000	.163	19.542	1.100	.288	24.449	3.045	.175	24.846	2.459
2.000	4.000	2.000	.150	21.090	1.040	.300	20.220	2.576	.150	24.846	2.347
4.000	6.080	4.000	.300	18.727	1.080	.600	19.477	2.200	.300	24.846	2.543
6.333	8.170	6.170	.400	19.406	.840	.900	19.694	1.601	.050	20.173	2.347
8.500	9.500	8.080	.450	16.470	.920	1.700	15.293	.959	.850	20.173	1.983
10.000	13.333	9.500	.520	19.542	1.000	3.100	13.119	.828	1.200	20.173	1.983
13.000	24.500	13.250	.550	20.493	1.180	6.550	23.960	.000	2.800	18.597	1.254
24.170	27.000	24.000	1.340	31.522	.560	7.750	22.787	.000	6.100	13.156	.000
27.080	30.000	27.000	1.300	30.871	.600	9.400	21.189	.000	6.600	10.609	.000
30.000	32.670	30.000	1.500	30.464	.480	10.400	20.781	.000	8.200	7.159	.000
32.000	33.250	32.750	1.700	30.193	.420	10.000	40.398	.000	9.000	1.127	.000
33.170	37.170	33.750	3.040	46.222	.340	12.200	40.751	.000	8.700	1.697	.000
	48.300	37.250							9.600	2.458	.000
		38.080							10.600	.000	.000
		39.000							10.600	2.295	.000
		40.000							11.400	.000	.000
		41.000							10.600	.000	.000
		41.500							11.000	1.317	.000
		48.333							10.800	.000	.000
		50.000							11.700	.000	.000
1	2	3	1			2			3		

Protein (g/l) vs Time (hours)

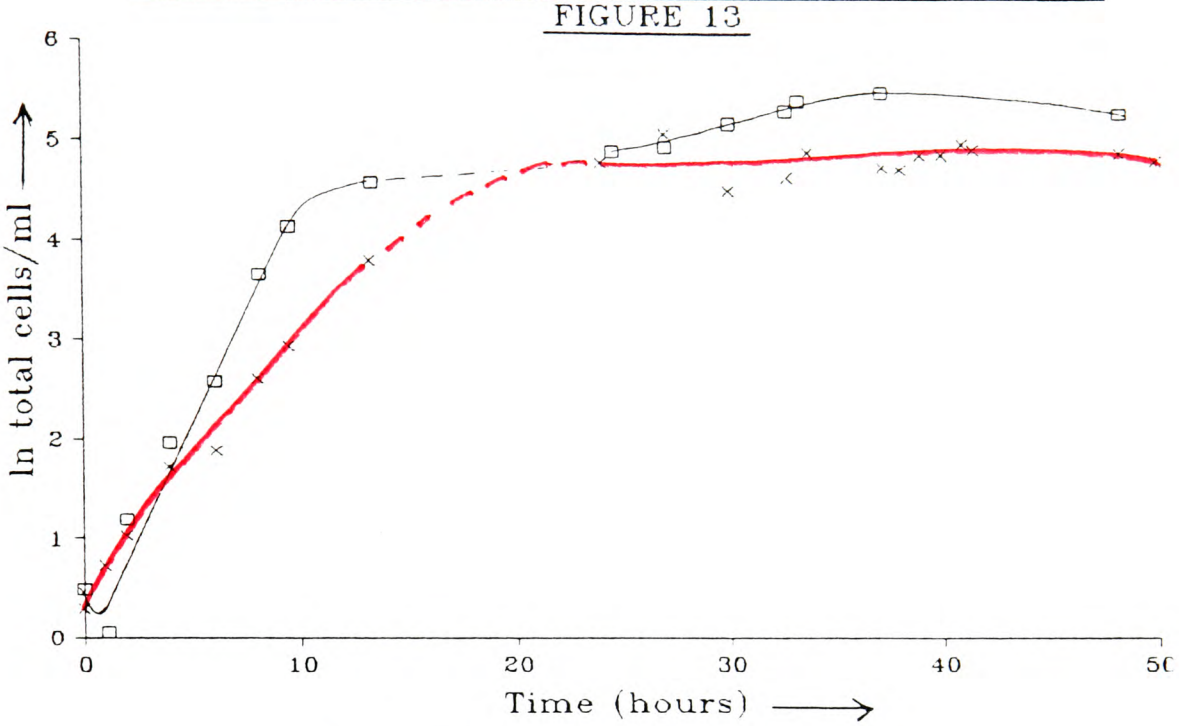
FIGURE 12



Time (hours)			Protein (g/l)		
.000	.000	.000	.052	.000	.000
1.000	1.080	1.000	.056	.000	.038
2.000	2.000	2.000	.081	.063	.000
3.333	3.080	4.000	.105	.140	.097
4.000	4.000	6.170	.081	.234	.242
6.333	6.080	8.080	.160	.315	.405
8.500	8.170	9.500	.148	.505	.615
10.000	9.500	13.250	.266	.654	.849
13.000	13.333	24.000	.354	.963	.904
24.170	24.500	27.000	.391	1.475	.988
25.500	25.670	30.000	.499	1.436	1.211
27.080	27.000	32.750	.554	1.499	1.156
28.500	28.500	33.750	.645	1.442	1.334
30.000	30.000	37.250	.680	1.507	1.283
33.170	32.670	38.080	.539	1.654	1.220
	33.250	39.000		1.573	1.483
	37.170	40.000		1.489	1.324
	48.333	41.000		1.336	1.410
		41.500			1.283
		48.333			1.179
		50.000			1.255
1	2	3	1	2	3

ln total cell count vs time (hours)

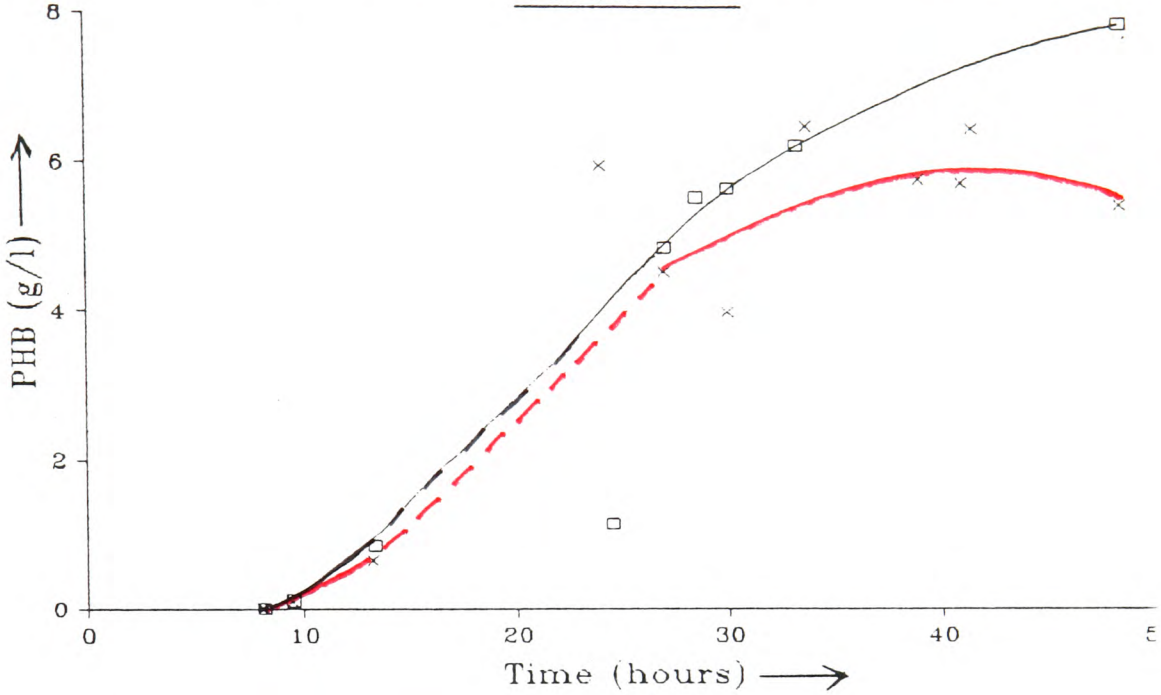
FIGURE 13



Time (hours)		ln cells / ml	
.000	.000	19.485	19.296
1.080	1.000	19.056	19.726
2.000	2.000	20.189	20.030
4.000	4.000	20.961	20.720
6.080	6.170	21.584	20.882
8.170	8.080	22.662	21.611
9.500	9.500	23.138	21.943
13.333	13.250	23.582	22.796
24.500	24.000	23.885	23.780
27.000	27.000	23.927	24.059
30.000	30.000	24.161	23.496
32.670	32.750	24.293	23.638
33.250	33.750	24.389	23.880
37.170	37.250	24.480	23.746
48.300	38.080	21.276	23.719
	39.000		23.867
	40.000		23.869
	41.000		23.978
	41.500		23.914
	48.333		23.884
	50.000		23.807
1.	2	1. □—□	2. ×—×

PHB formation vs time (hours)

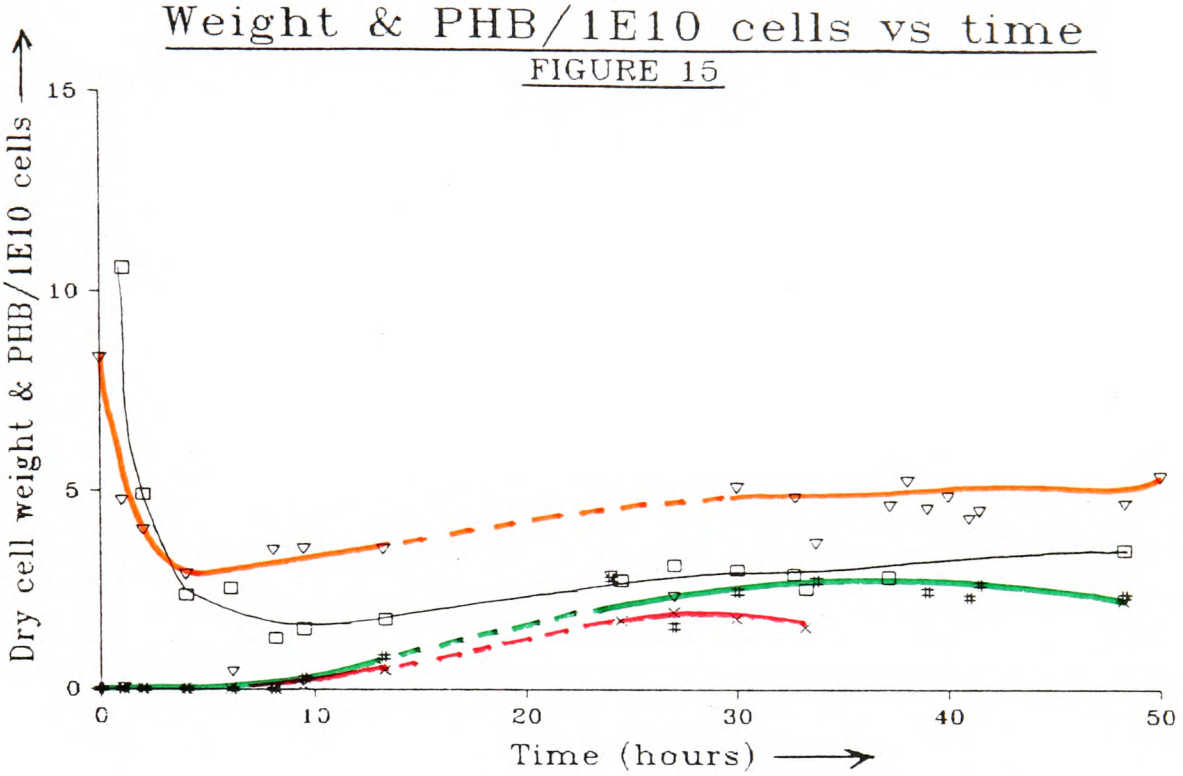
FIGURE 14



Time (hours)		PHB (g/l)	
8.170	8.080	.000	.000
9.500	9.500	.107	.095
13.333	13.250	.853	.656
24.500	24.000	1.145	5.943
27.000	27.000	4.850	4.516
28.500	30.000	5.518	3.982
30.000	33.750	5.640	6.478
33.250	39.000	6.215	5.774
48.300	41.000	7.866	5.718
	41.500		6.462
	48.333		5.432
1.	2.	1. □—□	2. ×—×

Weight & PHB/1E10 cells vs time

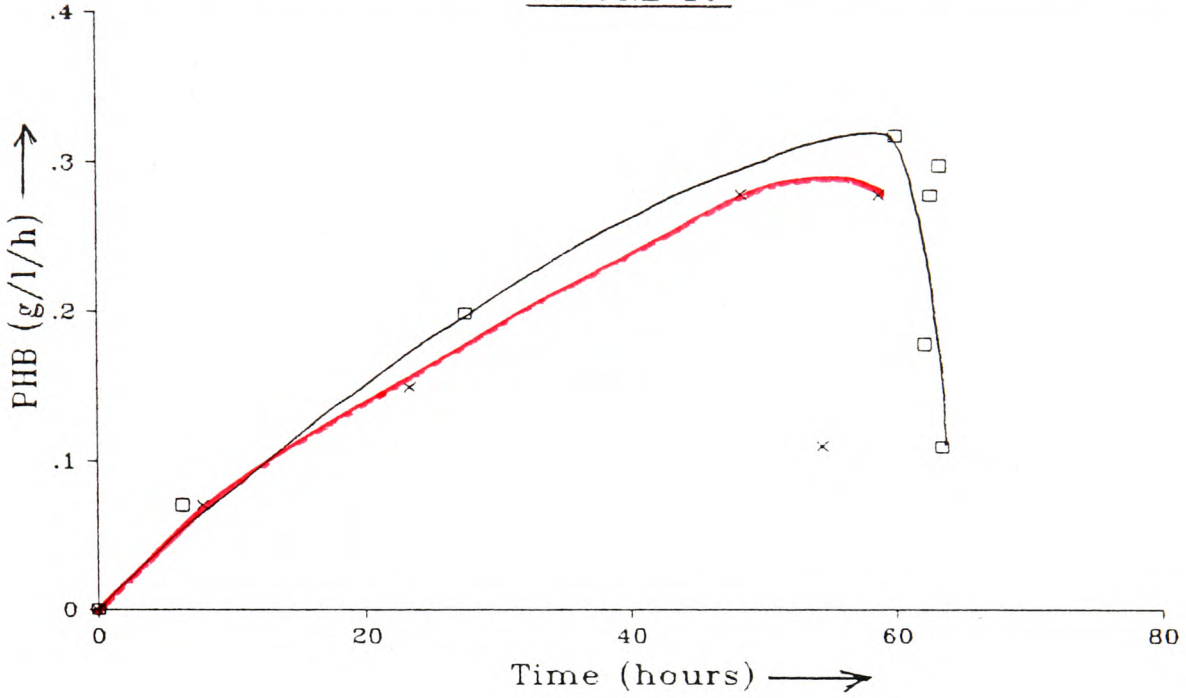
FIGURE 15



Time	(hours)	weight/ 1E10 cells	PHB/ 1E10 cells	weight/ 1E10 cells	PHB/ 1E10 cells
1.080	.000	10.590	.000	8.300	.000
2.000	1.000	4.900	.000	4.750	.000
4.000	2.000	2.360	.000	4.000	.000
6.080	4.000	2.540	.000	2.860	.000
8.170	6.170	1.290	.000	.430	.000
9.500	8.080	1.520	.100	3.500	.000
13.333	9.500	1.780	.490	3.540	.280
24.500	13.250	2.770	1.750	3.520	.830
27.000	24.000	3.150	1.970	2.870	2.800
30.000	27.000	3.020	1.810	2.350	1.610
32.670	30.000	2.930		5.120	2.490
33.250	32.750	2.560	1 590	4.880	
37.170	33.750	2.850		3.700	2.760
48.300	37.250	3.530	2.250	4.670	
	38.080			5.300	
	39.000			4.570	2.490
	40.000			4.910	
	41.000			4.340	2.340
	41.500			4.530	2.660
	48.333			4.720	2.370
	50.000			5.390	
1.	2.	1.		2.	

PHB formation (g/l/h) vs % dry weight

FIGURE 16



Time (hours)		PHB (g/l/h)	
.000	.000	.000	.000
6.300	7.870	.070	.070
27.500	23.400	.200	.150
60.000	48.420	.320	.280
62.150	54.500	.180	.110
62.600	58.800	.280	.280
63.280		.300	
63.400		.110	
1.	2.	1.	2.

Chapter 7

Section A - The ICI "CASE" award industrial session - copolymer production using mixed carbon feeds, in fed-batch experiments.

Section B - Industrial scale-up work; examination of two 0.5, 5 and 50M^3 fed-batch reactor processes.

Introduction

The link with **ICI** and the **Polytechnic of Wales'** current research, was started in 1984, one year prior to this author commencing work on the project. After some initial explanatory discussions, a "CASE" award (Collaborative Award in Science and Engineering) was granted, in addition to the normal **SERC** (Science and Engineering Research Council) studentship, in 1986. The regulations and guidelines of the award call for close collaboration between the industrial sponsor, and the associated academic institution. Financial support is given by the company, which covers an amount for an equipment budget (just over twice the **SERC** allocation of £400 p.a). Additionally, the company provides financial help for the researcher to travel to and from the company's research facility, and to relevant scientific conferences or meetings. The researcher is expected to spend at least six weeks every year, working with the

host company, in their research laboratories, on aspects of the project. Additionally, the company pays for the accommodation during this period. Thus, the industrial help, in financial terms, usually amounts to about £2500 p.a. The allocation of the additional equipment grant is usually crucial to the success of the project, due to the rising costs of research equipment.

Of immense value, was the chance to liaise with prominent scientists within industry, with a share of information vital. The extent of "trust" placed upon the researcher and academic institution, can be instrumental in determining industrial "data-sharing". Of course, this is a two-way system. If the company provides the best possible help to the researcher, then the benefits to the company are, in theory, much better. This is not necessarily dependent on a successful result! If results show a detrimental effect, then this can be as important as a new breakthrough. It would save the company duplicating the project, and deploying vast resources in the process.

In the case of this particular project, ICI had not already got an established commercial production system, where product was sold in other than trial circumstances. As a result, the project fitted in with part of their research investigative aims.

With the confines of this research agreed upon by both parties, the six-week industrial session work program was suggested. This was arrived at after a preliminary one-week examination of facilities, reactor availability, and current problem and idea information. As a direct consequence of this, three topics were identified for investigation, within the:-

Section A.1 - "CASE" award industrial session.

The three subjects were:-

(a) Polymer production, using fed-batch culture.
(b) Polymer recovery method; downstream processing.
(c) Polymer analysis, and analytical procedures for determining polymer percentage

Section A.1.(a) - Polymer production - fed-batch culture.

A compromise was arrived at for this part, as the research in this thesis concerns mainly pure **PHB**, whereas ←**ICI's** interest now lies mainly with copolymer production. As a result, five experiments were to be undertaken, which although making copolymer, would be relevant to pure polymer work. In addition, a bonus was being able to use both the production strain, ***Alcaligenes eutrophus*** **H/16 S301/TRON**, and a newly described mutant, designated **PRON** (which was developed from **TRON**). These were merely

company designations, not official taxonomic names.

A directly relevant part of the experiments, was being able to partially study the effects of temperature. It was hoped to answer the problem of scale-up specific growth rate loss, as well. In communication with **Bu'Lock (1988[92])** (Editor, Biotechnology Letters), it was suggested that the problem of initial scale-up (from shake-flask to reactor), could be attributed to removal of necessary CO_2. This in turn would effect the growth rate. Therefore, an experiment was conducted, with mass spectrometry of the exit gases, to investigate the effect of a 13% CO_2 inlet gas content. The precise nature of the experiments, designated **F5-BP7** to **F5-BP11**, were as follows:-

(i) F5-BP7.

Alcaligenes eutrophus **H/16 S301/PRON** was grown at 34°C, using medium 10, (**Chapter 4, Section B.1, Table 3**). As copolymer production was to be investigated, a mixed carbon feed was used, but only during the fed-batch stage. Initially, the bacteria were grown on glucose, to promote biomass. The mixed-feed carbon supply was a solution of 275g/l glucose and 160g/l propionic acid. This gives a glucose equivalent of 474g/l, and 41% of the carbon is derived from propionate. Propionate is not used initially, as it is toxic to the cells. In the polymer

storage phase it would be kept below 0.1g/l ideally, but it did rise to a maximum of about 0.5g/l. Concentrations above this, however, occurred at the very end of the run.

The bacteria were grown in a 3l batch reactor vessel, with a 2l working volume. Aeration was set to 0.3vvm air, whilst maintaining 50-80% DOT (relative to 100% air saturated solution). pH was controlled to 6.8±0.1, using HCl or NH_4OH (50% solution). The use of ammonium hydroxide also provided a nitrogen source. The limiting nutrient for use in all the five experiments was phosphorus, as H_3PO_4 (1.1M). A polypropylene glycol antifoam oil was used, with a timed dosage system (use of silicone antifoams had to be avoided, as it was very difficult to separate from **PHB**). Agitation was set to 500rpm throughout.

The final culture volume was 1700ml, which was acidified to pH 5.0, and stored at 4^oC until polymer recovery. The final biomass concentration was 39g/l, 68% of which was copolymer. The final duration of the experiment was 65 hours. The plot of results is shown on **Figure 17.**

(ii) F5-BP8.

F5-BP7 was repeated, and gave a cell density of 44g/l, 80% of which was copolymer, with a final cell

culture volume of 1700ml. The results are on **Figure 18.**

(iii) F5-BP9.

In this experiment, the **TRON** mutant was used, at an operating temperature of 37°C, as opposed to 34°C. The mixed carbon feed was slightly different, at 455g/l glucose equivalent, of which 39% of the carbon was derived from propionate. The actual mixture was 145g/l propionate and 279g/l glucose. In this experiment, which was done as a control (to see if **PRON** produced a better copolymer), the effect of temperature was also built-in. The experiment was conducted for 71 hours, in total. After 65 hours, the temperature was raised to 40°C for two hours. After the two hours, the temperature was raised to 42°C, and then again to 45°C. This was used to see what temperature **TRON** could survive at. It was also used to help the downstream processing, by promoting cell lysis and therefore facilitate easier extraction. A final cell biomass of 43g/l was reached, which had a 73% copolymer content. The results are shown on **Figure 19,** along with the gas analysis results on **Figures 24 and 25,** experiment 1.

(iv) F5-BP10.

The F5-BP9 experiment was modified by supplying a 13% CO_2 inlet gas content. This was used to answer the

scale-up query, using exit-gas analysis. The experiment was run for 72 hours, with no alteration of the set temperature (37^{o}C). A biomass concentration of 45g/l was reached, containing 75% copolymer. The results of glucose, dry weight and propionate concentration are shown on **Figure 20.** The gas analysis results are shown on **Figures 24 and 25,** experiment 2.

(v) F5-BP11.

A direct comparison between **TRON** and **PRON** was conducted, using **TRON** at 34^{o}C. Additionally, the feed rate was increased to a maximum of 30ml/hour, but later lowered to 15ml/hour. This was to see whether a faster utilisation was possible (without toxicity), which would reduce the experimental time, by encouraging faster storage. As a result, a final cell density of 51g/l was reached, after only 48 hours, with 75% copolymer content. The results of acetate, glucose and propionate concentration are depicted on **Figure 21.** Dry weight, **PHB** and **%PHB** of the cell dry weight are shown on **Figure 22.** **PHB** formation rate versus **%PHB** stored is depicted on **Figure 23.**

Section A.1.(a).1 - Reasoning behind, and evaluation of the experiments.

In the five experiments, no samples were taken from

inoculation until the end of the exponential growth. The only exception to this (in the last three experiments), was the record of exit gas composition. This was achieved by using on-line mass-spectrometry. The first two experiments were to have had gas analysis, but machine failure rendered this impossible. This was unfortunate, as a full comparison between **PRON** and **TRON** could then have been done. The other measured parameters included glucose, dry cell weight and **PHB** concentration, residual propionate (and occasionally acetate, using gas chromatography), carbon feed and alkali supply rates. Culture volume was recorded, as was pH and DOT. A cell yield (g cell/g glucose) was calculated throughout, as was the final product yield (1g biomass or **PHB** /1g glucose supply = Y_{cell} or polymer of 1.0). The analytical support provided at **ICI** was used to provide limited determination of Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, PO_4^{3-}, Mg^{2+}, K^{+}, and ammoniacal nitrogen.

The experiments were conducted for the following reasons:-

(i) **F5-BP7.** This was done to evaluate the effectiveness of **PRON's** accumulation of additional **HV** monomers. **PRON** was grown for selective tolerance to propionate. This organic acid, if incorporated into the fed-batch carbon feed, promotes methylation of **β-3-hydroxybutyrate** monomers (**HB**). The resultant monomer

is **β-3-hydroxyvalerate** (**HV**). The copolymer (**poly(HB/HV)**) is a more desirable product, for the commercial application of the **BIOPOL** polymer range. The **PRON** mutant was developed to broaden the **BIOPOL** range, by producing a higher mol% of **HV** in the copolymer. Reference to copolymer production was detailed in the paper of **Doi, Tamaki and Soga** (1987[6]). Their production allowed for up to 90 mol% **HV** in the copolymer, using a fed-batch carbon feed of 20g/l pentanoic acid. In reality, the properties of pure **PHB** (and pure **PHV**, if it were isolated), would themselves, separately, be commercially less important. By incorporation of **HV** into a monomer chain of **HB**, then the brittleness and elasticity of the product is radically improved. The specific application of each polymer, depends on the physical and chemical properties required for the plastic product. Thus, a system, such as **ICI's BIOPOL** range, would be more commercially useful having a wide spectra of copolymers. Particular blends can be made to order. Therefore, **PRON** was devised to improve the flexibility of the **BIOPOL** range. The aim of experiment **F5-BP7**, was to examine just how good **PRON** was at incorporating propionate into **HV** monomers, instead of degrading it and forming **HB**. Of crucial importance to the experiment, was the revertance rate of **PRON**, back to **TRON**. In practice, a test of genetic stability, was to show that **PRON** was indeed stable.

(ii) **F5-BP8**. This experiment (repeating **BP7**), was

done to check the repeatability of the results.

(iii) **F5-BP9.** In this instance, **TRON** was used to check just how good **PRON** was at forming **HV** monomers. By setting the experiment at 37°C, a direct comparison was not possible. The effect of temperature was also investigated towards the end of the experiment, by sequential temperature rises. It was highly unlikely, that full interpretation of temperature could be gained in this way. A more realistic experimental design would have been to carry out experiments at initial tempartures of 34, 37, 40, 42 and 45°C, and investigate each separately.

(iv) **F5-BP10.** This experiment was virtually a re-run of **BP9**, with one exception; that of inlet gas supply. It was hoped that the inclusion of 13% CO_2 in the gas supply, might settle the scale-up problem.

(v) **F5-BP11.** A direct temperature comparison was possible between **PRON** and **TRON**, as this experiment involved growing **TRON** at 34°C. It was also hoped to see whether the typical 65 - 72 hour duration could be reduced, using a faster carbon feeding rate.

The five experiments were therefore trying to address a number of questions. The time constraints and reactor availability, dictated the use of only five experiments.

This also allowed a week for the study of polymer recovery and analysis procedures.

Section A.1.(a).2 - Results of the ICI "CASE" award experiments.

Table 10

Experiment	F5-BP7	F5-BP8	F5-BP9	F5-BP10	F5-BP11
Temperature (^{o}C)	34	34	37-45	37	34
Initial glucose (g/l)	8.0	12.1	11.0	11.8	11.0
g/l glucose, dry weight and time at end of expon. phase	0.7 3.465 16.5hrs	0.65 5.3 16.0	0.3 5.4 16.0	2.0 5.27 24.0	2.5 4.09 16.0
Fed-batch feed composition -glucose (g/l) propionate (g/l) ≡glucose (g/l)	 280.0 159.0 473.0	 280.0 159.0 473.0	 280.0 145.0 455.0	 279.0 145.0 455.0	 279.0 145.0 455.0
% C from propionate	41.0	41.0	39.0	39.0	39.0
Max. feed rate (ml/h)	≡10	≡10	≡10	≡10	≡30(10)
Final dry weight (g/l) Copolymer (g/l) % of cell wt.	 39.0 26.6 68.0	 44.0 35.4 80.5	 43.0 31.6 73.4	 46.0 34.6 75.3	 51.0 38.26 75.0
Final reactor volume (l)	1.7	1.7	1.35	1.4	1.55
Cell yield: g cells/ g glucose equivalent (exponential)	0.48	0.46	0.50	0.54	0.48
(storage)	0.37-0.32	0.37-0.34	0.39-0.3	0.37-0.35	0.37-0.35
Product yield: g polymer/ g glucose	0.20	0.26	0.27	0.30	0.337
Experimental duration (hrs)	67.0	66.0	71.0	72.0	48.0

pH was controlled to 6.8±0.1 using 50% NH_4OH and HCl. The reactors were 3l ones, with a working volume of 2l. The aeration rates for all except **BP10** were 0.3vvm, 650ml/min air. **BP10** had 1l/min air and 0.167l/min CO_2.

To assess the effect of the experimental design, it was necessary to get information on the coploymers formed. To this end, the next phase of work was examined:-

Section A.1.(b) - Polymer recovery method - downstream processing.

Each ex-reactor culture solution was held at $4^{o}C$, at a pH of 5, until required. A variety of downstream processing techniques were examined, based on **ICI's** current technology. The process comprised of the following four steps:-

1) Enzymic digestion.

2) Detergent treatment.

3) Bleaching and boiling stage.

4) Recovery, by filtration or centrifugation.

A detailed breakdown of this process was given in **Chapter 4, Section D.1.** This section also includes the earlier technique of solvent extraction. The basic four-step process was carried out, with several variations. These were done to assess the suitability of the actual process.

(i) **F5-BP7.** The culture was heat-shocked at 130-140^{o}C, using steam, in a specially built rig. The

resultant flocs were collected. There was a problem, due to scale, in that losses were inevitable. On a large scale, the losses will be of a similar volume, but would represent a much smaller proportion of the material. The collected flocs were allowed to settle, and then the aqueous layer was siphoned off.

(ii) **F5-BP8.** Once again, the procedure for **F5-BP7** was repeated.

(iii) **F5-BP9 -> BP11.** Each culture was divided into two, and one aliquot was heat shocked to 100^{o}C. This was done using live steam in the laboratory, rather than in the pilot plant laboratory. The other was left unheated. Both were then centrifuged (separately) to remove the cell-free supernatant (culture medium), and resuspended in distilled water using a homogeniser. The heat-shocking was done to rupture cells, denature proteins (particularly depolymerases, which could do severe damage to released polymer granules) and facilitate easier extraction.

Section A.1.(b).1 - Assessment of the effect of heat-shocking, and the extraction process.

(i) **F5-BP7.** This heat-shocked, centrifuged and resuspended solution was divided into four equal volume aliqouts. These were treated as follows, with respect to

the detergent treatment step. The other steps were done normally.

one) 1% detergent, 70°C temperature treatment (control)
two) 1% detergent, 37°C temperature treatment.
three) 0.01% detergent, 70°C temperature treatment.
four) 0.01% detergent, 37°C temperature treatment.

This was done in order to see whether 1% detergent was a suitable concentration, and whether 70°C was wholly necessary.

After bleaching the polymer with peroxide, the solution was boiled for 15 minutes at 100°C. During the peroxide treatment, the polymer aggregated to form a golf-ball sized stringy mass, which was very rubbery. This caused serious fouling, and would have hindered bleaching, by reducing the surface area of available polymer to be bleached. Consequently, the flocs were broken up continuously (manually), in order to allow the magnetic stirrer fleas to operate properly. Due to the flocculation, the filtration was very much easier to do, and the time to form a "matt cake" was not recordable.

As the cell dry weight of **BP7** was 39g/l, and the copolymer content was 68%, the total recoverable polymer should be 26.6g/l. The culture volume was 1700ml, so the

total polymer content should be 45.22g. As there were four aliquots, each should have 11.305g polymer. The actual recovered polymer was:-

Table 11.

Polymer F5-BP7	g polymer (possible)	g polymer (actual)	% recovery
1. 1%, 70°	11.305	6.403	56.6
2. 1%, 37°	"	6.413	56.7
3. 0.01%, 70°	"	6.275	55.5
4. 0.01%, 37°	"	6.289	55.6

The excessive losses were solely due to the heat-shocking procedure. The point to note, was that there was very little difference between the four. In fact, the standard error of the mean ($SE_{(M)}$%) ammounted to less than 0.6%. This meant that, statistically, there was absolutely no significant difference between each system. Thus, the use of 0.01% detergent at 37°C, would be most economical, in terms of chemicals and heating energy costs.

However, the situation could change when each of the polymers were analysed. It may be the case, that some of the treatments damage polymer quality, reducing the molecular weight, for example.

(ii) **F5-BP8.** Two treatments were done. The

heat-shocked culture was enzyme treated, then divided equally into two portions. The first one was treated normally, and then the second one was recentrifuged and resuspended twice. In between this additional wash, it was again heated, to 70°C, using live steam. The bleaching treatment then used two volumes of hydrogen peroxide for one hour, and a further two for the second hour. Once again, flocculation caused severe problems. The filtered, dried and weighed polymer gave the following result:-

Table 12.

Polymer **F5-BP8**	g polymer (possible)	g polymer (actual)	% recovery
1. Double washed reheated, and double bleached	30.088	17.4	57.8
2. Control	30.088	17.5	58.2

(iii) **F5-BP9/10/11.** With all these experiments, no losses initially occured. Heat-shocking was done using live steam directly, into a beaker containing the solution. Only half of each culture was heat-shocked in this way, to 100°C. The two aliquots were centrifuged, resuspended and treated as normal, up to the bleaching stage. The double peroxide treatment and residual boiling were carried out. The polymer was filtered, dried and

weighed. In these cases, the bleaching led to much less floc formation. This was probably as a result of **TRON** forming less mol% **HV** in the copolymer, than compared to **PRON**. The results of the heat-shocked and untreated culture gave the following results:-

Table 13.

Polymer	g polymer (possible)	g polymer (actual)	% recovery
F5-BP9 treated	21.32	21.77	102.1
" untreated	21.32	21.07	98.8
F5-BP10 treated	24.253	25.82	106.1
" untreated	24.253	18.99	78.3
F5-BP11 treated	29.652	33.42	112.7
"	29.652	33.77	113.9

In cases where the recovery was greater than 100%, this indicated an error either in the dry weight calculation or polymer estimates, or both. The untreated **BP10** aliquot was reduced, due to an accidental discard of polymer, after one of the centrifugation steps. Once more, with exception of **BP10**, there was no significant difference on recovery performance (between treated and untreated aliquots). Therefore, heat-shocking was not necessary to get efficient polymer recovery, on this small laboratory scale. The emphasis on scale is of paramount importance. For a pilot plant reactor (or a production vessel), centrifugation of vast quantities of

liquid poses practical problems. Flocculation facilitates the use of large, slow running industrial centrifuges. Polymer analysis would again show whether or not heat-shocking (particularly at 130-140^{o}C) damaged polymer quality.

Including the variations shown previously, the extraction procedure was as described in **Chapter 4, Section D.1.** For small (laboratory) scale work, no heat-shocking is necessary. For pilot or production scale work, heat-shocking is indeed necessary.

Section A.1.(c) - Polymer analysis, and analytical procedures for determining polymer percentage.

Polymer analysis used several techniques:-

(i) **GPC** (gel-permeation chromatography).
(ii) **DSC** (differential scanning calorimetry).
(iii) **HPLC** (high performance liquid chromatography).
(iv) **MFI** (melt-flow index).
(v) **NMR** (nuclear magnetic resonance).

The method of determining polymer percentage in cells was an enzymic one, specially adapted by **ICI** analysts.

Polymer analysis, using the above techniques, was explained in **Chapter 4, Section D.2.**

(i) **GPC.** This gives the molecular weight of the copolymers, which will determine the usefulness of the plastic. All the polymers were high molecular weight polyesters, particularly **F5-BP8 - 11.** This showed that none of the treatments particularly damaged the polymer quality. A molecular weight of $1*10^6$ was generally very satisfactory, for reasons given in **Chapter 4, Section D.2.(a).** The results of this analysis were as follows:-

Table 14.

Polymer	Molecular weight		High or low
	Range	Average	
F5-BP7/1	$8*10^3-6*10^6$	$0.86*10^6$	High
F5-BP8/2	$1*10^4-9*10^6$	$1.05*10^6$	V.high
F5-BP9 treated	$2*10^4-7*10^6$	$1.09*10^6$	"
F5-BP10 treated	$1*10^4-7*10^6$	$1.04*10^6$	"
F5-BP11 treated	$1*10^4-8*10^6$	$1.01*10^6$	"
F5-BP11 untreated	$4*10^3-8*10^6$	$1.03*10^6$	"

(ii) **DSC.** This indicated the melt and recrystallisation points, and preliminary polymer quality. In the case of high mol% **HV** copolymers, recrystallisation was characteristically absent. Use of known, standard copolymers (with predetermined **HV**

content), enable a rough "standard-curve" to be plotted. The results of **DSC** are shown in the table below.

Table 15.

Polymer	Melting Pt (°C)	Crystallisation Pt (°C)	%HV
PV13	106.09	none	27.0
PV11	110.04	"	19.0
PV3	116.53	"	15.7
PV14	144.69	"	7.0
PO3	155.44	"	5.9
pure **PHB**	176.0	60	0.0
F5-BP7/1	124.24	none	unknown
F5-BP7/2	125.83	"	"
F5-BP7/3	123.32	"	"
F5-BP7/4	124.28	"	"
F5-BP8/1	131.09	"	"
F5-BP8/2	123.95	"	"
F5-BP9 treated	138.49	"	"
untreated	138.43	"	"
F5-BP10 treated	139.39	"	"
F5-BP11 treated	135.68	"	"
untreated	135.40	"	"

A plot of %**HV** (pure **PHB** as 0%, and standards) versus melting points was done. The best-fit curve of

0-19% **HV** was calculated statistically, and the actual figure had a correlation coefficient, r, of 0.99. This meant that the line was almost totally linear. This subsequently suggested the following preliminary mol% **HV**s, for the copolymers:-

Table 16.

Polymer	Mol% **HV**	Polymer	Mol% **HV**
F5-BP7/1	14.14	**F5-BP9** treated	10.08
F5-BP7/2	14.40	untreated	10.12
F5-BP7/3	13.69		
F5-BP7/4	14.13	**F5-BP10** treated	9.82
F5-BP8/1	12.19	**F5-BP11** treated	10.88
F5-BP8/2	14.22	untreated	10.96

In the original experimental design, it was hoped that **PRON** would use the propionate, rather than decompose it. Because it was more tolerant to the acid, a greater mol% **HV** content was envisaged in the copolymer, than compared to **TRON**. It was suggested that under these conditions, **PRON** would give somewhere in the region of 20 mol% **HV**, and **TRON** 10 mol% **HV**. The preliminary postulated results, were indeed indicative of the fact that **PRON** stored more **HV**, but not with the differential hoped for. This is likely to be a function of the inaccuracy of the standard curve, which was not really feasible. This was because of the nature of the copolymer; **HV** monomers are

incorporated at random. Two samples from the same batch may vary, depending on the chain composition, which will create problems in the validity of the standard curve. Thus, further determination was necessary, **HPLC** and **NMR** testing was required. Both **DSC** and **GPC** were qualitative tests, whilst **DSC** was used, albeit limited, as a quantitative test for mol% **HV**.

(iii) **HPLC**. This was also a quantitative test, which can show, once again, the mol% **HV** in the copolymers. The results of the analysis were as follows.

Table 17.

Polymer	mol% **HV**	Polymer	mol% **HV**
F5-BP7 (cells)	20.33	**F5-BP9/6**	10.995
F5-BP7/1	20.705	**F5-BP9/10**	9.98
F5-BP7/3	23.31	**F5-BP9**	10.15
F5-BP7/4	21.05	treated	
F5-BP8 (cells)	21.21	**F5-BP10**	10.33
		treated	
F5-BP11 treated	9.58		
untreated	10.89		

Problems were encountered when **DSC**, **HPLC** or **NMR** were done. This was shown in the results from **BP7**, which gave a range of values, indicating the nature of the copolymer chains inclusion steps. However, it

demonstrated the difference between **PRON** and **TRON**. **PRON** had typically 20-23 mol% **HV**, whilst **TRON** had 9.58-10.995 mol%.

HPLC was a good method for mol% **HV** analysis, the results were backed up by the **NMR** analysis. The actual procedure for forming **HPLC** samples was briefly given in **Chapter 4, Section D.2.(iii)**.

(iv) **NMR**. This was used for the same reasons as **HPLC**. The results of the analysis done at **Newcastle University**, and one done at the **Polytechnic of Wales**, were:-

Table 18.

Polymer	mol% ratio **HV/HB** by:-	
	methyl resonance	methylene resonance
F5-BP7/1	(24.3)	(26.7)
F5-BP8/1	18.2	18.8
F5-BP9 treated	12.5	14.0
F5-BP10 treated	10.6	11.7
F5-BP11 treated	13.2	14.0
F5-BP11 untreated	13.0	13.5
F5-BP8/1 (Poly. of Wales)	23.5	19.6

(**F5-BP7/1**, results inaccurate, and not used)

The preparation of **NMR** samples was again mentioned in **Chapter 4, Section D.2.(iv)**.

(v) **MFI.** This was a quality control technique. Extracted polymer was inserted into a heated chamber, melted and extracted under pressure. The time taken to extrude the polymer was recorded, as was the weight of polymer extruded after short intervals. For the copolymers produced, the following results were taken:-

Table 19.

Polymer	Melt-flow index (g)	doubling time (mins)	5g extrude time (mins)
F5-BP7/1	0.62	1.12	10.5
" /2	2.75	1.34	11.0
" /3	(0.09)	0.93	8.5
" /4	0.63	1.00	10.0
F5-BP8/1	0.59	1.57	12.0
" /2	0.11	0.98	12.5
F5-BP9 treated	0.40	1.85	13.5
" untreated	0.18	1.84	16.0
F5-BP10 treated	0.92	1.58	11.0
" untreated	0.42	1.66	12.5
F5-BP11 treated	0.94	2.24	14.0
" untreated	0.60	1.63	14.0

The melt-flow index and doubling time, have specific significance to polymer chemists and physicists. These figures would be used to gauge the suitability of a polymer. The time taken to extrude 5g of polymer also shows the state of the polymer. High molecular weight

polymers would be extensively cross-linked, and would take longer to extrude than a similar polymer of lower molecular weight. Details of the technique were given in **Chapter 4, Section D.2.(v)**. The interpretation of the results will not be covered, as it is outside the scope of the present work.

Section A.1.(c).1 - Enzymic determination of polymer.

The technique for enzymic analysis of polymer content of cells, was briefly described in **Chapter 4, Section C.2.4**. The problem with the technique, however, came when cells containing **HV** monomers were tested. Since the enzyme used was **D-3-hydroxybutyrate dehydrogenase**, full oxidation of the hydrolysed monomers into **acetoacetate** was hindered with **HV** monomers. The enzyme has a specific affinity for **HB** monomers. When **HV** containing cells were tested, the activity decreased. Consequently, during testing, three standard **PHB** weights were used. These were 3, 5 and 10mg of pure polymer, hydrolysed and analysed. The 10mg of extracted copolymer would give less absorbance than pure **PHB**. The decrease in absorbance is directly linked to **HV** content; as more **HV** was present, so the absorbance got less and less. Normally, samples for copolymer content determination are done before the mol% **HV** content is known. The results are therefore going to show less polymer than is actually present, when compared to similar samples containing pure **PHB**. The five standard

copolymers were tested, and the reduction in absorbance gave a calibration curve. This was used to gauge the actual percentage copolymer, to adjust the original figures once the **HV** content was ascertained. The calibration table of mol% **HV** content and resultant % polymer, along with the actual sample polymer results are depicted on the next page.

For the five copolymer standards, and all the unknown samples, 10mg of polymer/cells were used. In the case of copolymer, if the enzyme worked with **HV** as well as **HB**, then 10mg of pure copolymer should have given 100% polymer content. As **HV** was not attacked specifically by **D-3-hydroxybutyrate dehydrogenase**, the apparent polymer content was lowered. Plotting the mol% **HV** versus the apparent % polymer gave the calibration curve. This was linear, having a correlation coefficient, r, of 0.9528. As a result, if the mol% **HV** content was known, then a correction factor was used. For the 5.9 mol% **HV** coploymer standard, there was an apparent polymer content of 94%. The 6% **HV** gave a reduction of 6%. Thus, to get the true result, the apparent one would be multiplied by 106%. **F5-BP7** had a **HV** content of 21%, and the activity at this level was reduced by 13%. Thus, 10mg of **F5-BP7** had an apparent polymer content of 60%. Multiplying this by 113% gave a true value of 68% polymer. This was repeated throughout, to get the results. The multiplication factor was expressed as x 1.13 for 113%.

Table 20.

Polymer	mol% HV	% polymer by test	% polymer, corrected for HV content
(standards)			
PV13	27.0	86.83	100 (x 1.152)
PV11	19.0	87.83	100 (x 1.139)
PV3	15.7	92.21	100 (x 1.084)
PV14	7.0	94.17	100 (x 1.062)
PO3	5.9	94.24	100 (x 1.061)
pure PHB	0.0	100.00	100 (x 1.000)
(10mg cell samples, not pure polymer)			
(unknowns) (by HPLC)			
F5-BP7	21.35	60.22	68.11 (x 1.131)
F5-BP8	21.12	71.22	80.45 (x 1.130)
F5-BP9/6	10.995	60.21	64.60 (x 1.073)
F5-BP9/8	"	70.70	75.85 (x ")
F5-BP9/9	"	67.16	72.05 (x ")
F5-BP9/10	"	68.45	73.44 (x ")
F5-BP10/8	10.33	70.44	75.32 (x 1.069)
F5-BP11/2	10.24	9.58	10.24 (x ")
F5-BP11/4	"	34.13	36.48 (x ")
F5-BP11/6	"	49.05	52.43 (x ")
F5-BP11/7	"	59.11	63.18 (x ")
F5-BP11/8	"	66.83	71.43 (x ")
F5-BP11/9	"	69.90	74.71 (x ")
F5-BP11/13	"	70.19	75.02 (x ")

The problem of lower activity with copolymer

containing cells, led the **ICI** analysts to discontinue the technique, for copolymer containing samples. However, the author of this thesis, in work at **ICI, Billingham,** showed the relationship between mol% **HV** content and reduced activity. Therefore, samples could be done, and a provisional answer gained. Once the mol% **HV** content was known (using **HPLC** or **NMR**), then the true value of the polymer content could be calculated. Thus the technique was validated for use with copolymer containing cells.

Section A.1.(c).2 - Discussion of the results of the CASE award experiments, experimental analysis techniques and polymer recovery procedures.

The first two experiments were done to demonstrate the high **HV** incorporation of the **PRON** mutant's polymer. The postulated figure for mol% **HV** inclusion was 20%, roughly twice that expected of **TRON**, under similar conditions.

The growth rate of the bacteria in these two experiments was not determinable, as no optical densities were recorded. No cell counts and dry weights were measured, until the beginning of the stationary phase, when polymer storage would have started. For **F5-BP7**, there appeared to be a lower inoculation volume than **F5-BP8**. At the end of the exponential growth, the dry weight was 3.46g/l, as opposed to 5.3 (**Figures 17 and**

18). At the inoculation stage, **BP7** had an excess glucose concentration of 8g/l, which decreased to 0.7g/l. For **BP8**, the excess carbon was 12.1 initially, which had decreased to 0.65g/l after exponential growth (**Figures 17 and 18**). If a two-hour lag phase is assumed, based on earlier findings, then **BP7** utilised glucose at a rate of 0.53g/l/hour. **BP8**, conversely, used glucose at 0.82g/l/hour. This indicated that the inoculation for **BP7** was low, and glucose would have been used from 7-16 hours. The long lag phase is indicative of a low inoculation level.

The final product formation of both **BP7** and **BP8**, was lower than any of the **TRON** runs, in terms of polymer yield from carbon supplied. The low inoculation problem of **BP7**, meant that as both **BP7** and **BP8** were of similar duration, **BP8** had a higher cell density, and a greater polymer content of cells. In fact, the % **PHB** of the dry weight was highest in **F5-BP8** than any other experiment.

As regards the polymer formed itself, using the general enzymic/ detergent/ bleaching treatments, improvements on the system were not significantly seen. However, from **BP7**, it was noted that the use of 0.01% detergent and 37^{o}C, was as good as 1% detergent and 70^{o}C. This is obviously important from an economic point of view. It is likely, however, that the mixing of large quantities of cells (as after a production run), would

require the higher concentration and temperature. This would probably facilitate the best treatment. Both **BP7** and **BP8** polymers had a high molecular weight, with a relatively low melting point. The discovery that they had a mol% **HV** content of 18-21%, explained this low temperature. In all extraction procedures, **BP7** and **BP8** yielded very light, rubbery material, which was hard to break-up.

The advantage of using **PRON**, in getting a high mol% **HV** content, was shown by the **TRON** experiments. **F5-BP9** also had a temperature investigation built into it. Apart from the initial temperature of 37°C being different, the effect of raising the final stage temperature would not be conclusive. The increase in temperature, however, prompted a slight increase in CO_2 production. This was seen when the temperature was raised from 37-40°C only. Increases above 40°C decreased the CO_2 liberation. The result was exactly mirrored in the oxygen uptake, which increased once again. During the storage phase, up until these temperature changes, the level of CO_2 and O_2 remained essentially constant. It is suggested that further experiments be carried out, on different initial temperatures. This would give a clearer, more conclusive picture.

The amount of biomass formed was less than **BP8**, as was the amount of polymer stored. The reduction in mol%

HV, would also have been exacerbated by a reduction in % carbon derived from propionate. Both **F5-BP7** and **BP8** had a carbon feed with 41% of the carbon derived from propionate. **BP9-11** had only 39% derived from propionate. This discrepancy would not account for the reduction in **HV** content, which in **BP9-11** was 10-13%. All the **TRON** copolymers had a very high molecular weight, with a melting point about 10°C higher than the **PRON** copolymers.

With **BP9-11**, the copolymers produced were much less rubbery, indicative of the lower **HV** content. It was interesting to note, that heat-shocking of **BP9-11** did not affect the polymer, in terms of molecular strength. This was indicated by almost exactly the same melting points, for both heat-shocked or untreated polymer. However, a physical change was brought about, by 100°C heat-shocking of these lower mol% **HV**-containing copolymers. The heat-shocked polymers were not so rubbery as their untreated counterparts, and broke-up easier. This would have helped the bleaching stage of extraction. **F5-BP10**'s copolymer had the lowest **HV** content, apparently, on a physical basis. This was backed-up by evidence from the NMR results, but not supported by HPLC results.

Whilst experiments **F5-BP9** and **BP10** were being run, mass spectrometry of the exit gases was carried out in full. For **F5-BP9**, when the experiment was started, very quickly CO_2 liberation and O_2 uptake increased (**Figures**

24 and 25, experiment 1). With **BP10**, however, 13% CO_2 was included in the gas supply. This was to hopefully investigate the scale-up problem hypothesis. A direct comparison between **BP9** and **BP10** was made more difficult, as in addition to imposing a CO_2 influx, the aeration rate was increased from 650 to 1000ml/min. However, a plot of exit CO_2 and O_2 gave a good indication of the bacterial responses. With **BP10**, the increase in CO_2 production and O_2 utilisation was not seen until 14 hours (**Figures 24 and 25**, experiment 2). It then lasted for 24 hours, when exponential growth would be coming to an end. By examining the linear portions of the plots, the results achieved were as given in the next table. In addition, plotting the ln (natural log) of the difference (between initial and sampled values), gave specific growth rates for the two runs. These are also included in **Table 21**, on the next page.

From the table, it is seen that putting in a 13% CO_2 supply does not encourage faster growth. In fact, if the growth rate of **BP9** (based on O_2) was achieved in **BP10**, then the oxygen uptake would still be a lot less than **BP9**. It was hoped to show whether or not the provision of extra CO_2 would benefit the bacteria. **Bu'Lock (1988[92])** suggested that the reason for a lowered growth rate (between shake-flask and 1l fermenter experiments), was due to possibly CO_2 degassing of the reactor. This would limit the exponential growth. Even

Table 21.

Experiment	F5-BP9	F5-BP10
Reactor working volume (l)	2	2
Gas supply rate	650ml/min air	1000ml/min air, 167ml/min CO_2
composition (%)		
O_2	21	18
CO_2	0	13
O_2 uptake rate	0.808 10-14 hours, C of r=0.982	0.165 17-24 hours, C of r=0.981
CO_2 formation rate (%/hour)	0.781 10-14 hours, C of r=0.979	0.187 17-24 hours, C of r=0.187
Specific growth rate (μ, hours)		
ln O_2 vs time	0.370 0-14 hours, C of r=0.996	0.124 0-24 hours, C of r=0.97
ln CO_2 vs time	0.341 0-14 hours, C of r=0.995	0.104 0-24 hours, C of r=0.961

allowing for the fact, that a 50% increase in air rate was put on between **BP9** to **BP10**, the inclusion of 13% CO_2 should have coped with this theoretical problem. Either 13% CO_2 was too much, or, as was most probable, the "degassing of reactor CO_2" was not the reason. The most likely explanation, was due to Fe^{2+} precipitation with pH control. This was due to the levels of $FeSO_4.7H_2O$ used in the media investigated, were only marginally above the limiting concentrations required. With acidic medium, as before pH control, Fe^{2+} stays in solution. With the addition of KOH, iron precipitation limits the bacteria, so that the growth rate was lower. This was explained and justified in **Chapter 5, Section B.2.**

For the last experiment, **F5-BP11**, a faster feed rate was employed. This cut down the experimental duration to

48 hours, as opposed to 66-72 hours. The polymer storage was very good, to 75% of the total cell dry weight, which was the highest of the experiments, at 51g/l (**Figure 22**). The plot of the rate of **PHB** formation vs %**PHB** of the cell dry weight of **BP11** was interesting (**Figure 23**). The maximum rate of accumulation was 1.75g/l/hour, which was reached at a **PHB** concentration of 50-55% of the dry weight. This was a similar percentage to the results seen in **Chapter 6, Section C**, but the rate was significantly higher. Indeed, this was higher than **Sonnleitner, et al** (1979[23]) found, whilst using the parent strain. The accumulation rate was therefore "adjustable", by altering the feed rate. However, the amount of polymer stored, can not exceed a specific limit, without a drop in accumulation rate. The initial cell yield was more or less equivalent to the other experiments, although lower than the two "hot" experiments (**BP9 - 10** done at 37°C, **BP11** done at 34°C). Because of the temperature difference, this experiment was a very useful way to compare directly **PRON** and **TRON**. **BP11**'s polymer was, once again, a very high molecular weight one. From DSC results, it had a slighly lower melting point than **BP9** and **BP10**, indicating a possibly higher **HV** content. The results of HPLC and NMR were not conclusive for this, however. The increase in feed rate, from 10 to a 30ml/hour maximum (and then back to 10ml/hour), meant that a significantly higher product yield was achieved, at 0.34g polymer/g glucose equivalent. This was some 25

and 12% better than **BP9** and **BP10**, respectively. What was noticeable with **BP11**, was the excess glucose and propionate levels (**Figure 21**). These were higher than had previously been seen. 0.3g/l propionate was present in the medium after 44 hours, which rose to 1g/l at the end of the experiment. It was likely that the accumulation of **PHB** would have rapidly ceased beyond this time, due to toxicity. Excess glucose was present during the storage phase, at about 2g/l. What probably gave the likeliest indication of imminent cell death, was the presence of free acetate in the medium. After 48 hours, the shut-down point, supernatant acetate was at a concentration of 0.36g/l (**Figure 21**). This large amount had occured over the last 1½ hours of the experiment, and indicated a shift in metabolism. The dry weight and polymer accumulation were increasing well until 40 hours (**Figure 22**). The increase was still obvious after 48 hours, although the accumulation of polymer (as a % of the dry weight) failed to increase significantly in the last eight hours. With the sudden presence of free acetate, it was probable that the cells were drastically changing. The cells would probably have started to lyse, therefore, after this time. What was interesting to see, however, was that a rapid feed of propionic acid can be tolerated by **TRON** (and probably also by **PRON**). Practically, this means that experimental time can be shortened, which would increase the economic viability of the system, by reducing operating costs by one day.

The use of **PRON** was, therefore, eminently suitable for producing a higher mol% **HV** copolymer. In conjunction with the scheme proposed in **Chapter 10, Section 2, PRON** and **TRON** could be used successfully in a fully variable production. The **BIOPOL** range could then be adequately tailored for a wide range of applications.

The analytical techniques examined in this work were satisfactory, with a number of limitations. GPC was a very good qualitative method. DSC was useful for a quick determination of mol% **HV** content in copolymers, but was best used in a qualitative mode. HPLC and NMR were very good quantitative methods, but HPLC could also be used qualitatively. The enzymic analysis method was fully ratified for use with copolymers, and the extraction method was comprehensively investigated. In general, improvements to the production scheme were not significantly better. This would not be true, however, for bench-scale work, where the technique could be made more economic. Overall, the work done was a very good exercise, in which a tremendous amount of information and experience was gained.

Section B - Industrial scale-up work; examination of two 0.5, 5 and 50M^3 fed-batch processes.

An invitation to help out with two production runs

was extended to the author, by **ICI**. Apart from the plant staff, there were two **ICI** personnel (**D.Southgate, K.Richardson**) and a placement student from **Teeside Polytechnic**. K.Richardson and the Polytechnic student worked the day shift, with the author and D.Southgate working on the night shift. The work involved monitoring and data presentation, by taking samples from the reactor, gas analysis and chromatography.

The process involved seeding a 500l reactor, and growing it for 16-20 hours. The initial glucose was 5g/l, which decreased to 1g/l at the end of the exponential stage. The dry weight increased to 2g/l, and CO_2 reached 1.2% This was similar to the results of the CASE session experiments. The optical density was measured every two hours, and the operating temperature was 37°C, with a pH of 6.8 (±0.1, using 28% NH_4OH solution). The medium had $(NH_4)_2SO_4$ initially in it, and the limiting nutrient for **PHB** production was phosphate. The aeration rate was 0.3vvm air.

The $0.5M^3$ vessel was used to inoculate a $5M^3$ reactor. This was grown-up again for 16-24 hours, without nutrient limitation. The dry weight (at the end of the exponential phase) was 6g/l, and 3.8% CO_2 was produced. This was used to inoculate the $50M^3$ production vessel. The process reactor was used to produce the four tonnes of copolymer, containing 7 mol% **HV** content.

The $50M^3$ vessel was initially filled with $12M^3$ medium, which was seeded with the contents of the $5M^3$ vessel. Growth proceeded for 6-8 hours, when excess glucose decreased from 20 - 1g/l. The cell dry weight was about 8g/l. The initial feed regime was carried out for 8-12 hours. This was a solution of 460g/l glucose, which was fed in at an initial rate of 150l/hour. $5M^3$ of this glucose solution was added in total.

The final feeding regime fed in a mixture of 55g/l propionic acid and 376g/l glucose. The total volume of feed was $28M^3$, which gave a final volume of $50M^3$. The loading of glucose and propionate was such that only 13% of the solution was propionate. This was only one-third of that used in the CASE session experiments. In addition, **TRON** was used to produce the copolymer, and hence lower **HV** content. The last stage would last 50-60 hours. The final dry weight was roughly 110g/l. The glucose supplied to the system was 13 tonnes, which produced 5.5 tonnes of biomass, containing 73% copolymer (4.4 tonnes). The cell yield (Y_{cell}) for the 0.5 and $5M^3$ reactors, was 0.5 and 0.43 respectively. The final cell yield (Y_{cell}) of the $50M^3$ reactor was 0.42, with a polymer yield (Y_{pol}) of 0.31. These were similar to the previous results in this chapter.

The contents, once reacted, were discharged to a collecting vessel, cooled to 15^oC, and pHed to pH 5. The

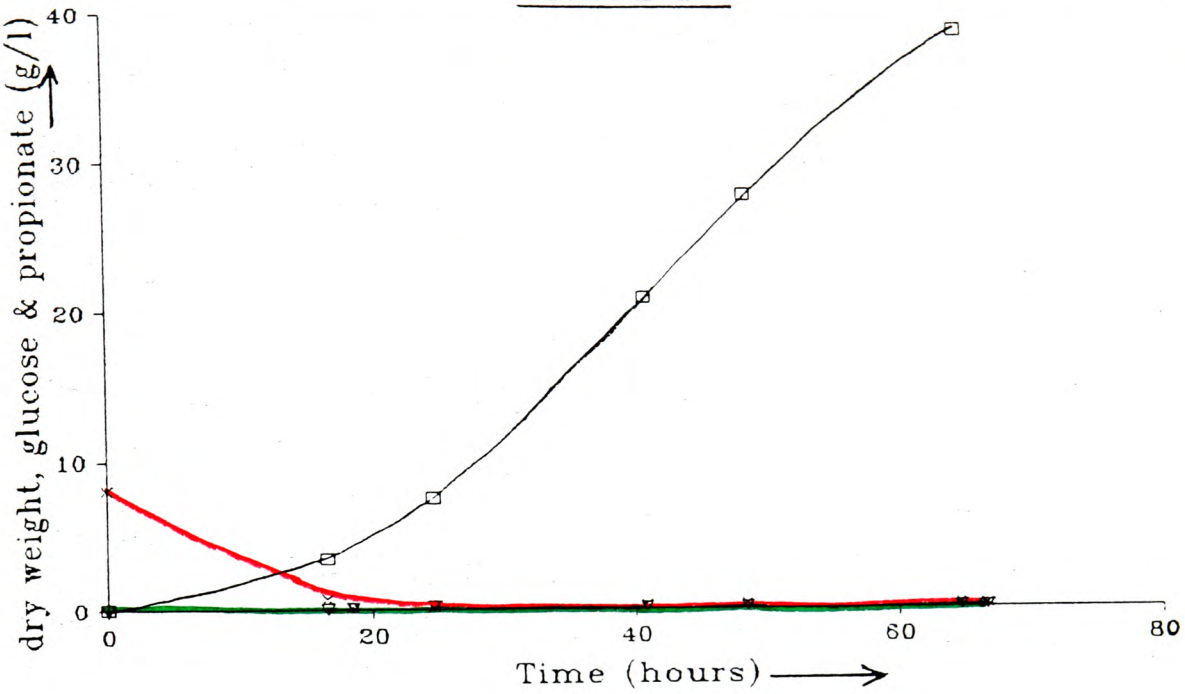
product was tankered to the recovery plant, for the conventional recovery technique. The final reaction time was about 110 hours, but varied between four to seven days, due to non-reaction procedures.

The parameters that the author measured were exit gas composition, aeration rate, temperature and pH. The volume of residual feed was also recorded. A gas chromatograph was also used to analyse for excess propionic acid concentration. Glucose analysis was done by taking samples, centrifuging them and measuring supernatant glucose quantity (using an automatic enzyme analysis machine). Optical density readings were done by the plant staff. Before each inoculation, sterility testing was carried out visually, and indirectly using a nutrient broth enrichment technique. The results were plotted as they were gained, before handing on to the next shift.

The experience gained with these processes was very useful, an invaluable insight into production-scale processes. In the first run, the author was requested to check for a possible gram +ve contaminant. Apart from this, it transpired that the pH control was faulty, and a high KOH concentration was reached. Consequently, the samples looked like soapy water. The second run was a complete success, fortunately. The chance to work on such a large scale was much appreciated.

dry weight, glucose & propionate vs time

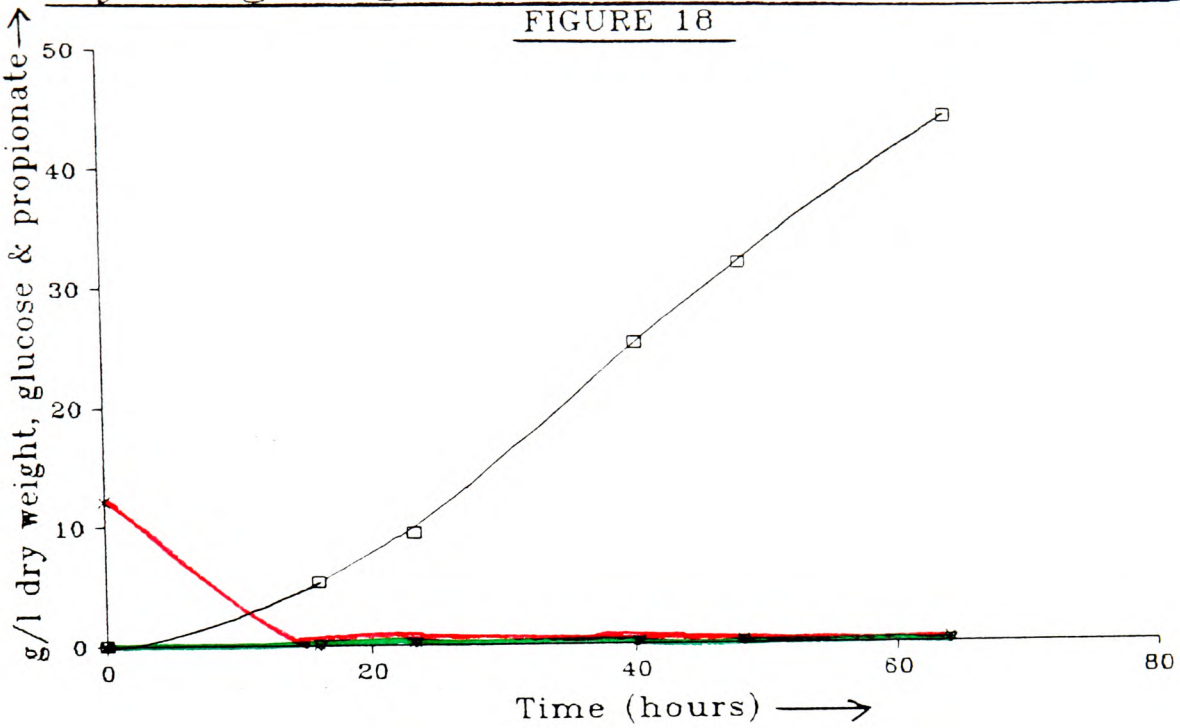
FIGURE 17



Time (hours)	Dry cell weight (g/l)	Glucose (g/l)	Propionic acid (g/l)
.000	.000	8.000	.000
16.583	3.460	.700	.000
18.500		.100	.100
24.667	7.530	.100	.180
40.833	20.970	.240	.100
48.500	27.860	.300	.100
64.750	39.000	.300	.100
66.750		.200	.100

dry weight, glucose & propionate vs time

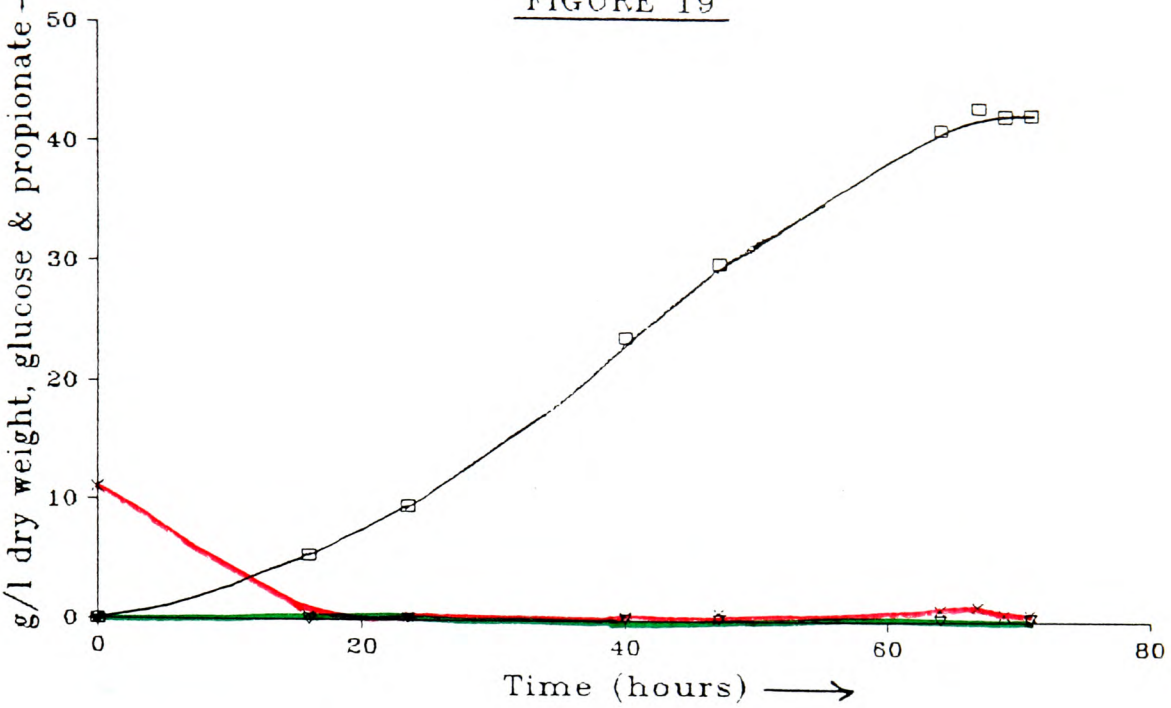
FIGURE 18



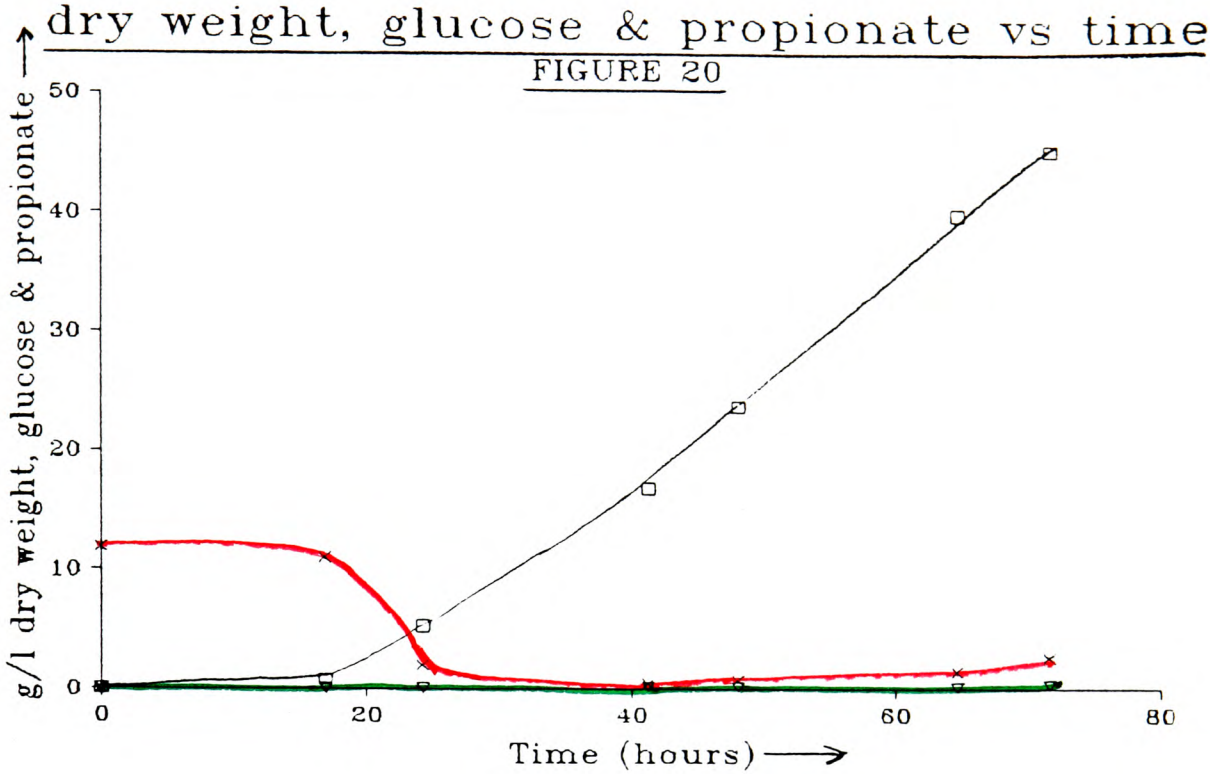
Time (hours)	Dry weight	Glucose	Propionic acid
.000	.000	12.100	.000
16.083	5.300	.050	.000
23.333	9.440	.200	.100
40.250	25.340	.200	.100
48.250	31.960	.350	.100
64.083	44.080	.400	.100

dry weight, glucose & propionate vs time

FIGURE 19



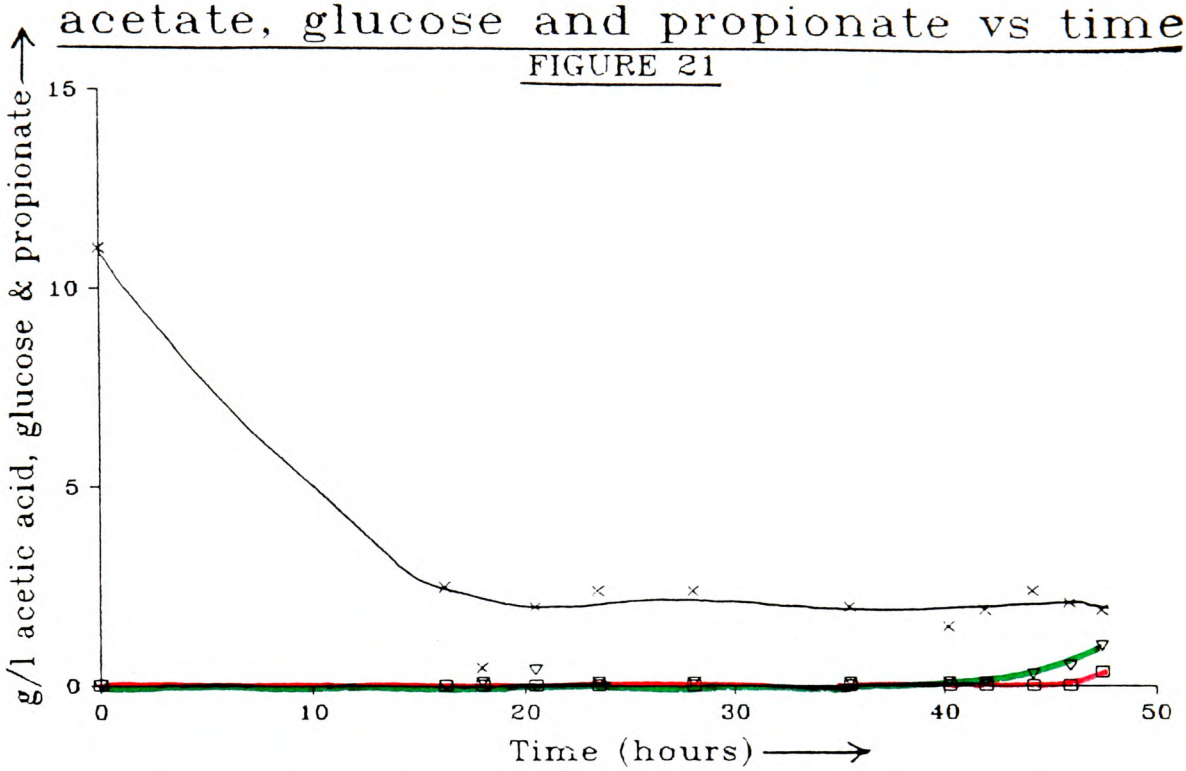
Time (hours)	Dry weight	Glucose	Propionic acid
.000	.000	11.000	.000
16.000	5.395	.300	.000
23.500	9.570	.200	.100
40.000	23.915	.300	.100
47.167	30.070	.600	.100
64.083	41.570	1.000	.100
67.000	43.515	1.200	
69.000	42.820	.600	
71.000	42.920	.600	.250



Time (hours)	Dry weight	Glucose	Propionic acid
.000	.000	11.900	.000
16.917	.740	11.000	.000
24.250	5.270	2.000	.000
41.250	16.900	.450	.100
48.083	23.800	.700	.100
64.750	40.100	1.400	.100
71.750	45.500	2.600	.350

acetate, glucose and propionate vs time

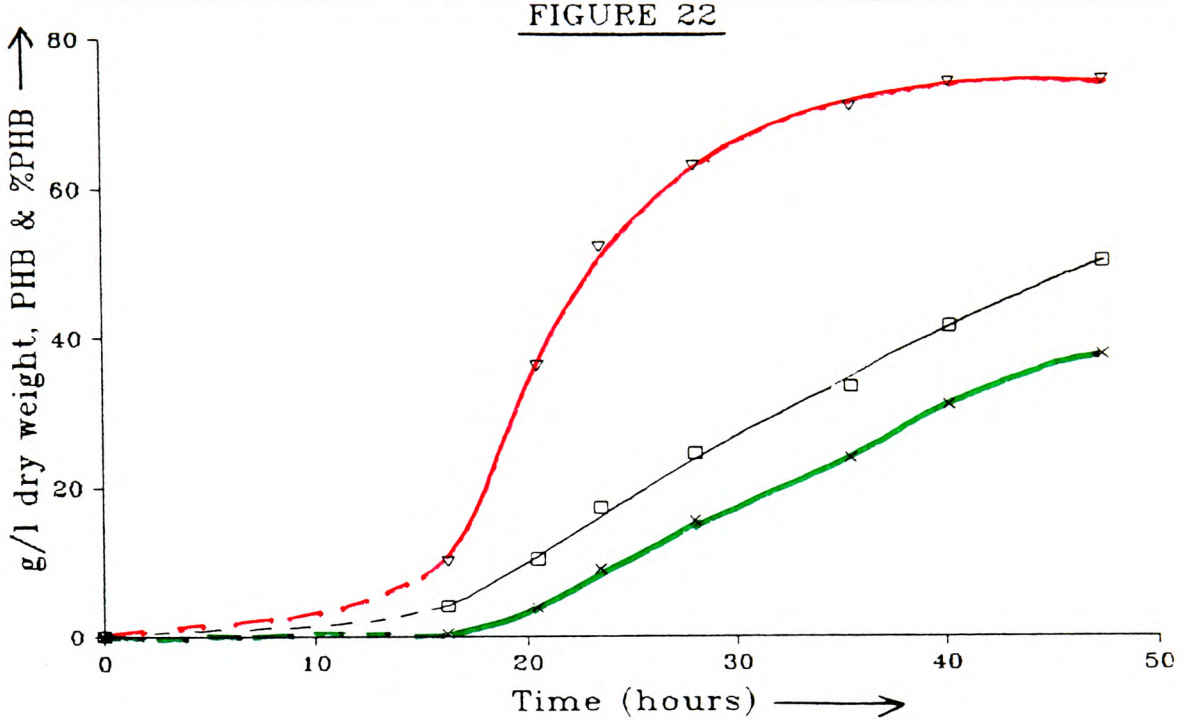
FIGURE 21



Time (hours)	Acetic acid	Glucose	Propionic acid
.000	.000	11.000	.000
16.250	.000	2.500	.000
18.000	.000	.450	.100
20.500	.000	2.000	.400
23.500	.000	2.400	.100
28.000	.000	2.400	.100
35.500	.000	2.000	.100
40.250	.000	1.500	.100
42.000	.000	1.900	.100
44.250	.000	2.400	.300
46.000	.000	2.100	.500
47.500	.360	1.900	1.000

Dry weight, PHB & %PHB of dry weight

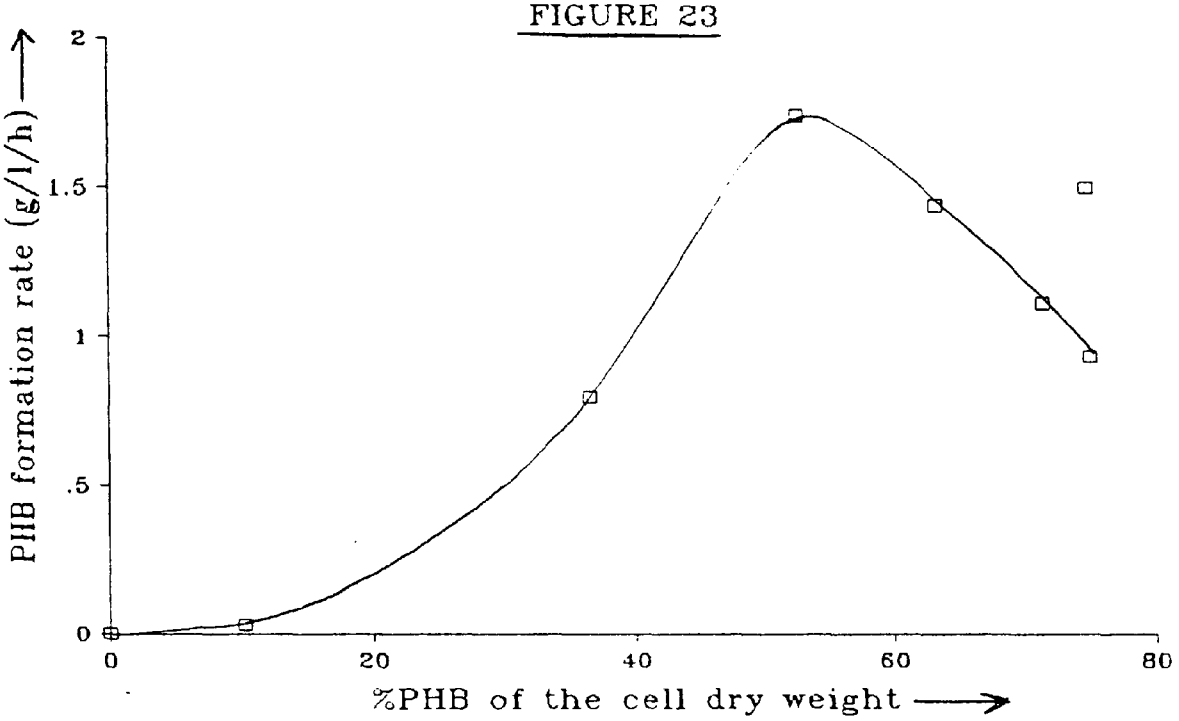
FIGURE 22



Time (hours)	Dry weight	PHB (g/l)	% PHB
.000	.000	.000	.000
16.250	4.090	.419	10.240
20.500	10.510	3.834	36.480
23.500	17.330	9.086	52.430
28.000	24.730	15.624	63.180
35.500	33.690	24.065	71.430
40.250	41.800	31.229	74.710
47.500	50.660	38.005	75.020

PHB formation rate vs %PHB stored

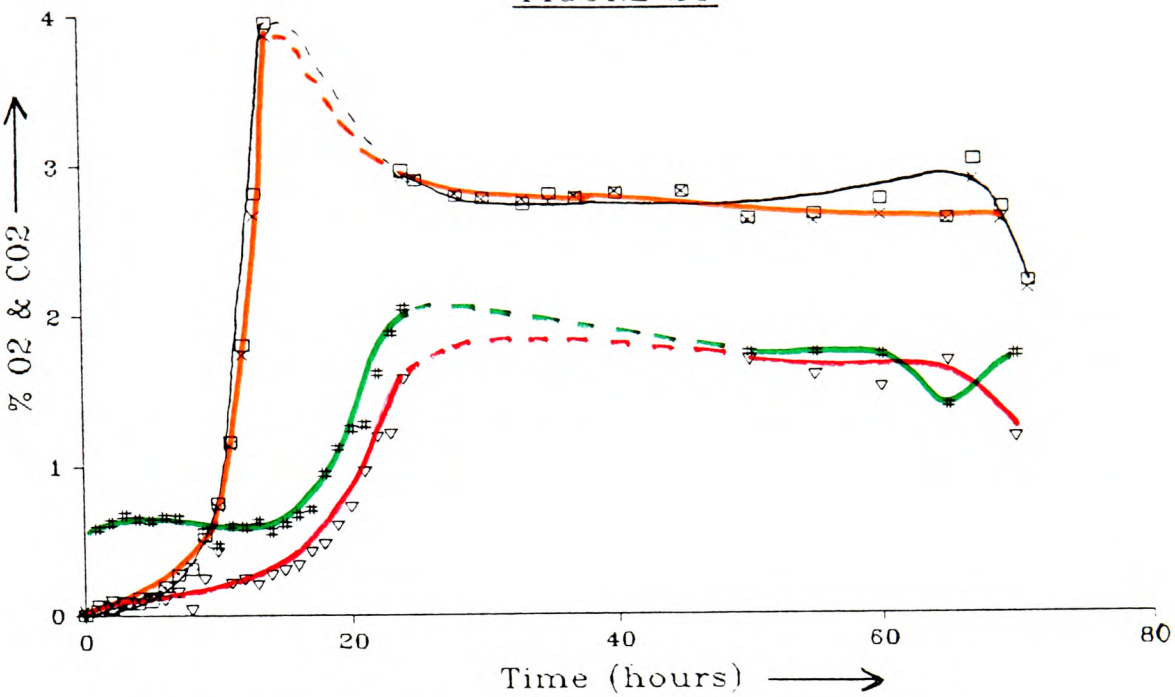
FIGURE 23



%PHB of the cell dry weight	PHB formation rate (g/l/h)
.000	.000
10.240	.030
36.480	.800
52.430	1.750
63.180	1.450
71.430	1.120
74.710	1.510
75.020	.940

O2 uptake and CO2 liberation, versus time

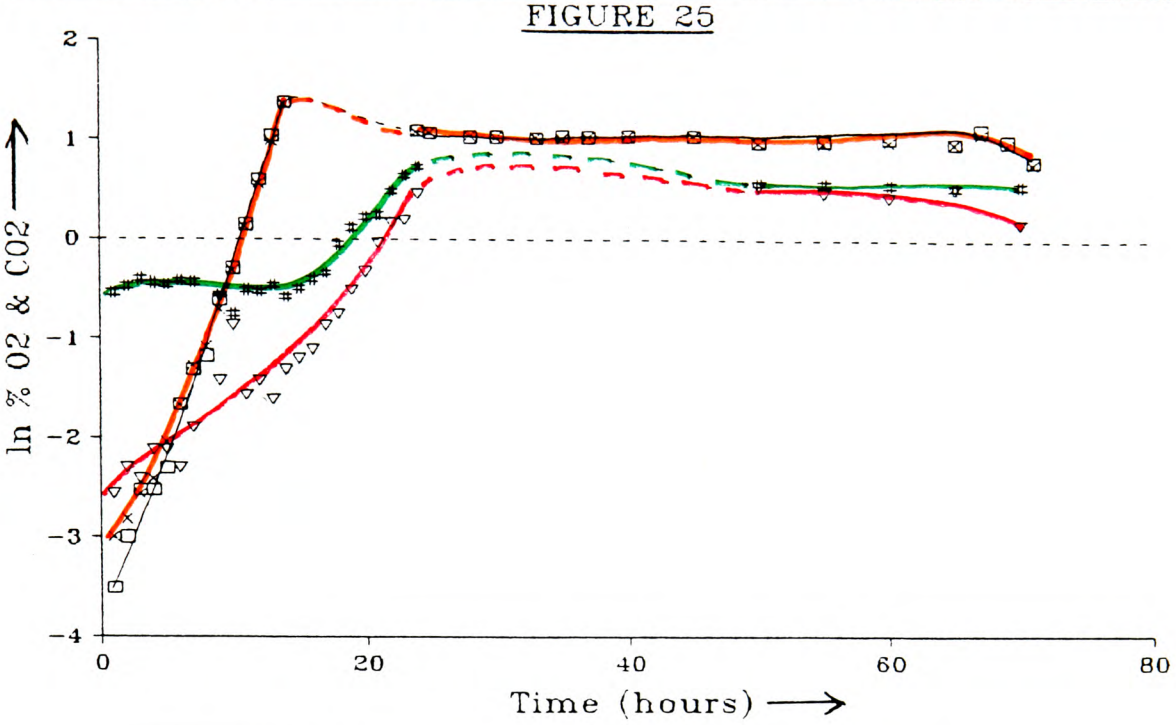
FIGURE 24



Time (hours)		Oxygen uptake %		Carbon dioxide liberated % .	
.000	1.000	.000	.030	.070	.580
1.000	2.000	.030	.050	.100	.620
2.000	3.000	.050	.060	.090	.670
3.000	4.000	.080	.070	.120	.640
4.000	5.000	.080	.090	.120	.630
5.000	6.000	.100	.130	.100	.660
6.000	7.000	.190	.190	.150	.650
7.000	8.000	.270	.280	.030	
8.000	9.000	.310	.340	.240	.570
9.000	10.000	.540	.490	.420	.470
10.000	11.000	.750	.730	.210	.600
11.000	12.000	1.160	1.140	.240	.590
12.000	13.000	1.810	1.740	.200	.630
13.000	14.000	2.820	2.670	.270	.560
14.000	15.000	3.960	3.870	.300	.610
24.000	16.000	2.970	2.930	.330	.670
25.000	17.000	2.910	2.920	.420	.710
28.000	18.000	2.800	2.820	.470	.950
30.000	19.000	2.790	2.780	.600	1.120
33.000	20.000	2.750	2.780	.720	1.250
35.000	21.000	2.820	2.770	.960	1.280
37.000	22.000	2.790	2.800	1.190	1.620
40.000	23.000	2.820	2.830	1.210	1.890
45.000	24.000	2.830	2.830	1.580	2.060
50.000	50.000	2.660	2.640	1.700	1.760
55.000	55.000	2.690	2.640	1.600	1.760
60.000	60.000	2.790	2.680	1.520	1.740
65.000	65.000	2.660	2.650	1.690	1.400
67.000	70.000	3.050	2.920	1.180	1.740
69.000		2.730	2.640		
71.000		2.230	2.180		
①	②	①	②	①	②

ln O2 uptake & CO2 liberation, versus time

FIGURE 25



Time (hours).		ln O_2 uptake.		ln CO_2 liberation.	
.000	1.000		-3.507	-2.569	-.545
1.000	2.000	-3.507	-2.996	-2.303	-.478
2.000	3.000	-2.996	-2.813	-2.408	-.400
3.000	4.000	-2.526	-2.569	-2.120	-.446
4.000	5.000	-2.526	-2.408	-2.120	-.462
5.000	6.000	-2.303	-2.040	-2.303	-.416
6.000	7.000	-1.661	-1.661	-1.897	-.431
7.000	8.000	-1.309	-1.273		
8.000	9.000	-1.171	-1.079	-1.427	-.562
9.000	10.000	-.616	-.713	-.868	-.755
10.000	11.000	-.288	-.315	-1.561	-.511
11.000	12.000	.148	.131	-1.427	-.528
12.000	13.000	.593	.554	-1.609	-.462
13.000	14.000	1.037	.982	-1.309	-.580
14.000	15.000	1.376	1.353	-1.204	-.494
24.000	16.000	1.089	1.075	-1.109	-.400
25.000	17.000	1.068	1.072	-.868	-.342
28.000	18.000	1.030	1.037	-.755	-.051
30.000	19.000	1.026	1.022	-.511	.113
33.000	20.000	1.012	1.022	-.329	.223
35.000	21.000	1.037	1.019	-.041	.247
37.000	22.000	1.026	1.030	.174	.482
40.000	23.000	1.037	1.040	.191	.637
45.000	24.000	1.040	1.040	.457	.725
50.000	50.000	.978	.971	.531	.565
55.000	55.000	.990	.971	.470	.565
60.000	60.000	1.026	.986	.419	.554
65.000	65.000	.978	.975	.525	.536
67.000	70.000	1.115	1.072	.166	.554
69.000		1.004	.971		
71.000		.802	.779		
1.	2.	1.	2.	1.	2.

Chapter 8.

Chemostat work; continuous culture as an alternative means to create biomass and PHB.

Section 1 - Introduction.

The previous chapters have concentrated on examining batch and fed-batch cultivation. In these cases, the biological reactor was filled with medium, cells were introduced and growth proceeded. The only additions, in the case of batch growth, were air, antifoam and alkali. With exception of the gaseous phase, the system was a closed one (with no exit for the culture). Once inoculated, essentially no new additional input occured. As a result, growth took place until such time as there was a toxic build-up, or nutrient limitation. Once either of these occured, growth ceased, and when cultivation continued, the cells eventually died. In the case of fed-batch, however, there was additional input, in the form of extra glucose. This was added as growth came to an end, which promoted the synthesis of **PHB**. Thus, in these two instances, which were both ultimately closed systems, batch and fed-batch cultivation led to biomass and **PHB** formation, respectively. In an industrial system, a series of batch reactors have to be used, to provide biomass for a fed-batch production stage. From an economic point of view, a system that provided a

continual stream of **PHB**-containing cells, would be desirable. This would make recovery a better proposition, if automated around a continuous process. Additionally, removal of a large proportion of "downtime" would be possible. This is the time taken to shut-down, empty, clean, sterilise, refill and reinoculate a batch reactor. Continuous culture requires much less downtime, by nature of it's operation. To this end, continuous culture was examined, to look at the conditions necessary to generate **PHB** and/or biomass.

Continuous culture, as the name suggests, allows for long-term growth of microbial cultures, be they plant, animal, yeast or bacteria. In order for such a long-term process to proceed, unless medium was continually added, growth would cease. As a result, continuous culture, done in a chemostat, relies upon the provision of a perpetual supply of fresh medium. This takes place after an initial batch growth process. The batch growth would be necessary to provide sufficient biomass, with which to proceed to long-term cultivation. If the chemostat was inoculated during the medium supply stage, then the cell population would not increase sufficiently. This is because the chemostat is an open system. As there is a flow of medium inwards, unless a very large reactor was used, the vessel would fill. Thus, the flow of medium in, when at equilibrium, would be balanced by flow of medium out. A weir or dip-tube is usually used to remove excess medium,

to a preset volume. The usual equation encountered with continuous culture is:-

$D = F/V$ where F = the flow of medium (into and out of the reactor), in l/h.

V = the working volume of the reactor, in l.

D = the dilution rate, hours.

If the volume and flow rate remain constant, then the reactor will have a specific dilution rate. This has a precise effect on the cultivation of cells. This stems from the fact, that in the medium used, the dilution rate figure (hours) cannot exceed that of the specific growth rate figure (μ, hours). This relationship stems from the equation:-

$$V.dx = V.\mu x.dt - Fx.dt$$

where x = biomass

V = volume of culture, l

F = flow rate, l/hour

μ = specific growth rate of biomass

dx = rate of change in biomass

dt = " " " in time

This describes the **increase in biomass**, which equals the **growth - loss occuring with outflow**. If the

process attains a steady state, where $dx/dt=0$, then $D=\mu$. Thus, the specific growth rate can be altered, by adjusting the flow rate, which will consequently alter D. If the flow rate is increased, so that D becomes greater than μ, then the rate of increase in biomass will be less than growth can provide, due to the large outflow rate. As a result, the reactor would lose more cells than it supported making, and eventually all the cells would be lost. This condition is termed "washout". For continuous culture, a range of dilution rates can be achieved, which will provide a range of specific growth rates. There is a maximum specific growth rate, μ_{max}, which corresponds to D_{crit}, the critical dilution rate. If $D > D_{crit}$, then washout will occur.

The use of different dilution rates allows the study of microbial cells, under different simulated environmental conditions. If the specific growth rate is fixed at a very low level, by reducing the flow rate and thence lowering D, then the effect of a limiting environment can be examined. As the flow of medium would be very low, the growth of the cells would also be slower, due to nutrient limitation. Unlike batch growth, this type of nutrient limitation can be sustained, to examine the effect of environmental change. If a cell was growing maximally, and then encountered nutrient limitation in it's natural environment, then various changes would be seen. The use of chemostats to study

environmental or ecological changes, the molecular, genetic and biochemical alterations, is a rather traditional one. The incorporation of continuous culture into established or new industrial processes is more recent.

If the chemostat could be used to control the growth rate, then the formation of **PHB** would seem an ideal system with which to use it. After growing the chemostat culture in batch, to get a good biomass, then the cells (grown under a high dilution rate in the reactor) could provide a constant stream of biomass. This might then be fed to another vessel, operated at a much lower dilution rate, which would simulate nutrient limited conditions. In the second vessel, the situation would be ideal for **PHB** formation. In this way, the system would use two different sized reactors. If the biomass was cultivated at a dilution rate 0.4hours, with a flow of 4l/hour, into a 10l working volume reactor, then a second reactor (producing **PHB**) would operate at a dilution rate of 0.1hours. The flow from the first would constitute the flow to the second, so to get the reduction in dilution rate, the volume would be increased, to a 40l working volume vessel. At this point, the term "residence time" can be introduced, which corresponds to D^{-1}. This term is frequently used in process engineering, to describe the time that a mass flowing into the reactor would stay, before flowing out. For the first reactor, a 0.4 hour

dilution rate would have a residence time of 2.5 hours. The larger vessel, would have a residence time of 10 hours. In batch and fed-batch work, residence time is important. The longer the cells are left in the reactor, with additional carbon, the more **PHB** is produced. For product formation, in the case of **PHB**, a longer residence time is required, than compared to biomass formation.

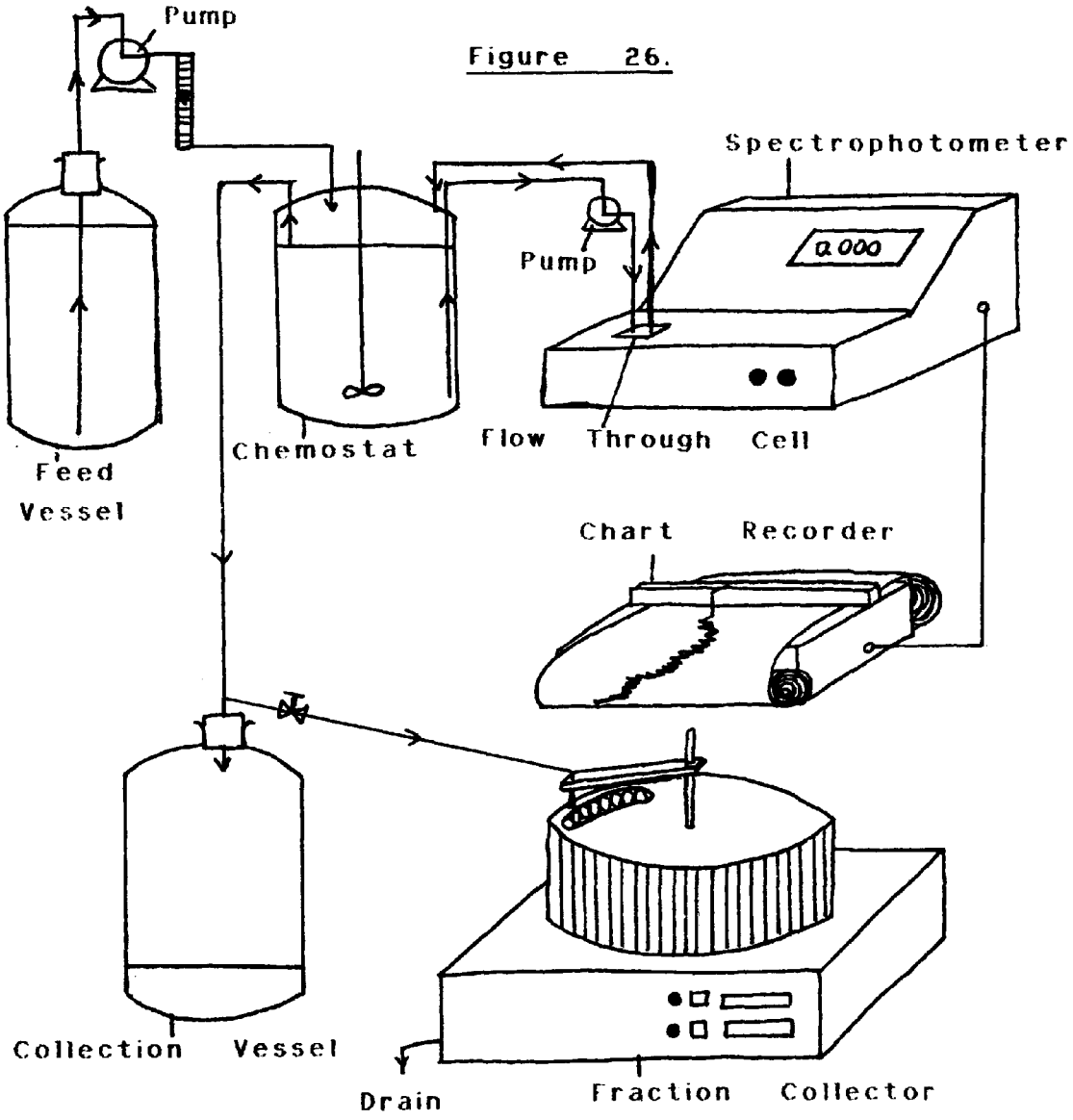
It was hoped to describe in these experiments, whether continuous culture could be operated solely, or whether a system of continuous and fed-batch multiple vessel configuration would be necessary.

Section 2 - Continuous culture experiments.

Two series of continuous culture experiments were examined. The results gained from this work were of secondary importance, when compared to the experience of experimental methods gained. Two experiments were conducted, the first of which was terminated due to excessive wall growth and foaming. Foaming in continuous culture is particularly damaging, as this can significantly decrease the volume, and therefore increase the dilution rate. If high dilution rates are used, then foaming can lead to washout before the apparent D_{crit} value is attained. The second experiment was terminated due to lack of medium and time. It was originally planned to run the continuous culture, from batch start-up,

examination of various dilution rates and then washout, all in one. In reallity, this was not possible. In fact, the maximum D rate used was below the washout and D_{crit} values. The experiments themselves lasted for 87 and 65 hours. The set up for fermentation, data-recording and sample collection was:-

Figure 26.



In this way, the initial batch growth could be assessed on-line. The overnight range (collected in the fraction collector) measured data on the effect of change

on dilution rate, leading up to steady state (taken as several unchanged readings). Operationally, two problems were encountered. Firstly, the fraction collector broke down, so that medium containing cells overflowed. Secondly, the flow through cell became blocked, due to cell clumping and excessive foaming. A 1l **LH** fermenter reactor was used, with the medium in **Chapter 4, Section B.1, Table 3.** The aeration rate was set, so that a dissolved oxygen tension of 80% was achieved. As the cells grew, the stirrer speed was adjusted manually, to cope with the additional oxygen required. Rapidly, however, the DOT fell below 80%, and reached a minimum of 10%. With only a 1l/min maximum air supply, and manual adjustment of stirrer speed, the control was not satisfactory. The specific growth rate (μ) of the two experiments was low, at 0.24 and 0.15h^{-1}. The results gained were not accurate enough, certainly above a dilution rate of 0.17h^{-1}. For this reason, the second series was run.

The **Polytechnic of Wales** experiments were run for 30, 105, 125 and 50 hours (the results of which are shown on **Figure 27,** as parameter versus dilution rate). Medium 5 was used (**Chapter 4, Section B.1, Table 3**), in a 1l **LH** vessel. 60% DOT was maintained, using a special control module. This recorded the DOT, and adjusted the stirrer speed accordingly. This gave very good control. At the start of the batch stage, the DOT was 100%, with a

correspondingly low stirrer speed. As the cell mass increased, and DOT decreased to 60 %, the speed controller was set into action (the DOT varied by typically ± 5%, from 60%). There were problems, however, due to air locking of the feed line, and a ***Bacillus*** contamination. The specific growth rates (μ) were better, at 0.23-0.29h^{-1}, but these were still lower than the shake-flask experiments.

For the first experiment, two dilution rates were achieved, 0.05 and 0.1h^{-1}, in the 30 hour duration of the run. In the second experiment, dilution rates of 0.06, 0.1 and 0.15h^{-1} were examined. This experiment proceeded for 105 hours, although contamination occured around 80 hours. This was four or five hours before a medium bottle was changed over. Initially, it was thought that the ***Bacillus*** species was introduced when the change over occurred, as this was the only compromise to sterility. However, it was found, through examination of samples prior to the change over time, that the ***Bacillus*** had "invaded" earlier. After 105 hours, the culture was pink, and very gelatinous. It was carefully sterilised and discarded safely. The samples up to 77 hours were examined only.

The third experiment operated for 125 hours, with the examination of dilution rates equal to 0.077, 0.144 and 0.286h^{-1}. The samples from the final 40 hours were not

used, as air locking had reduced the flow, and dilution rate as a result.

The final experiment examined the use of dilution rates of 0.318 and $0.353h^{-1}$. In previous batch experiments, $0.353h^{-1}$ would have equalled the fastest specific growth rate seen in the batch reactors. The shake-flasks were always higher, with the only exception being the non-pHed batch reactor, which had a specific growth rate of $0.54h^{-1}$. Washout in this final experiment was to be arranged, but was not possible, due to loss of medium. However, examination of the results showed that washout was fairly imminent, above a dilution rate of $0.35-0.4h^{-1}$

The second series of continuous culture, gave the following results. Biomass and **PHB** formation decrease with increases in dilution rate. The maximum **PHB** accumulated was 20-25%, occuring up to a dilution rate of $0.1h^{-1}$. Above this, the accumulation rapidly declines. For dilution rates up to $0.1h^{-1}$, the cells grew very slowly, accumulating **PHB** and protein. Above a dilution rate of $0.15h^{-1}$, the cells contained much less **PHB**, protein and dry weight, indicating rapid cell division. The residual glucose concentration rises linearly with increased dilution rate, from 1.5g/l at $0.05h^{-1}$, to 16.5g/l at $0.353h^{-1}$. Using this as a gauge for washout, and having an initial concentration of 20g/l, the

critical dilution rate would be around $0.4h^{-1}$. The dry weight and protein concentrations would also decline to zero at a dilution rate of $0.4h^{-1}$. The log of cell absorbance results, indicate that above a rate of $0.35h^{-1}$, they would very rapidly decrease. For dilution rates of $0.25h^{-1}$ and above, the residual $(NH_4)_2SO_4$ concentration builds up rapidly. With an initial concentration of 3g/l, the maximum dilution rate would be $0.35-0.4h^{-1}$.

Using continuous culture, **PHB** production was not really feasible, with this bacteria and medium. The maximum concentration of **PHB** occured between a dilution rate of $0.075-0.1h^{-1}$. At only 25% of the cell dry weight, this was merely 15% higher than the level seen in exponentially growing cells. It was much lower than that previously seen in fed-batch culture. Because of the slow specific growth rate, and the low productivity, it is not suitable for producing **PHB**. It is probable that the destiny of continuous culture lies within a multiple-stage system; whereby biomass would be generated for fed-batch production stages.

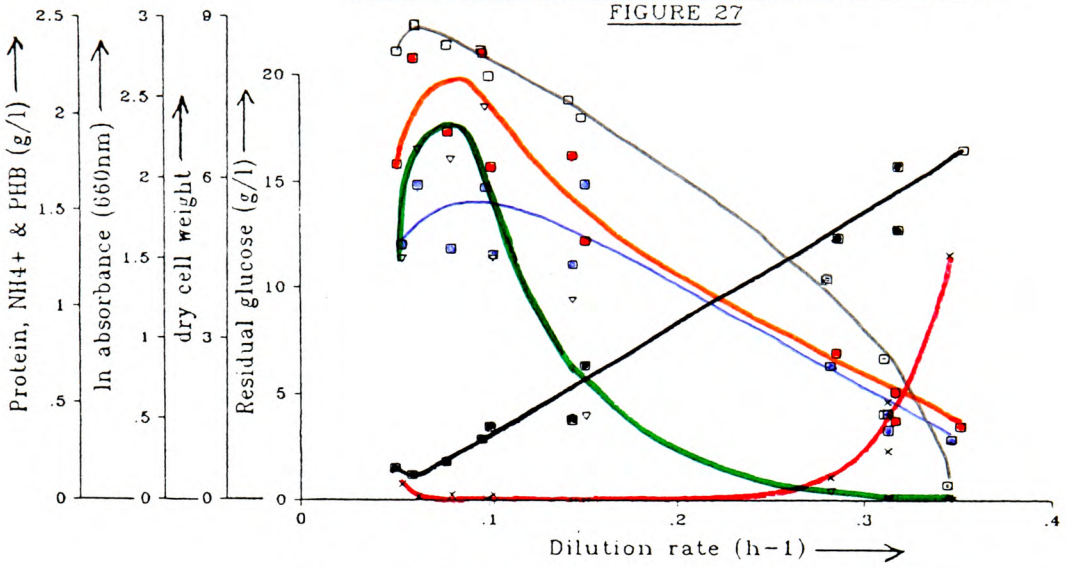
The amount of analysis done with continuous culture, was limited; detailed kinetic analysis was not done. This was because the production of **PHB** was required only. Since it is probably not suitable for this purpose, detailed investigation was not warranted, within the

confines of research time available. It was originaly planned to look at the kinetics of the system, in some detail, and model them with a software package (such as **ACSL** or **ISIM**, in conjunction with **Dr John Ingham, Bradford University**). The data from the **Polytechnic of Wales's** results are shown in **Figure 27**, at the end of this chapter. This is in the form of parameters expressed versus dilution rate.

In the final results chapter, multiple-stage fed-batch will be considered, as a process to produce optimum quantities of **PHB**, in a cyclical manner. This will be further developed, by use of continuous culture feeding biomass to a fed-batch production stage. This will form a semi-continuous process. An industrial model will be postulated and costed, in **Section 2, Chapter 10**, the **Conclusions**. This will follow other general conclusions of this work, found in this thesis. After the model, a comparison between the multiple-stage and continuous culture will be done, assuming high biomass and productivity in both. This will demonstrate the relative potential efficiencies, and compared to the actual findings.

Protein, NH4+ & PHB (g/l),
ln absorbance (660nm), Dry cell weight (g/l),
residual glucose vs dilution rate

FIGURE 27



Dilution rate, h-1	Residual glucose	Dry cell weight (g/l)	ln abs (660nm)	Protein	NH4+ (g/l)	PHB (g/l)
.050	1.510	6.300	2.800	1.330	.080	1.254
.059	1.180	8.270	2.960	1.640	.020	1.825
.077	1.790	6.910	2.840	1.310	.030	1.773
.096	2.870	8.370	2.810	1.630	.010	2.047
.100	3.460	6.260	2.650	1.280	.030	1.260
.144	3.800	6.470	2.500	1.230	.000	1.042
.151	6.340	4.870	2.390	1.650	.000	.436
.286	12.370	2.760	1.370	.700	.120	.047
.318	12.760	2.030	.880	.450	.250	.010
.318	15.730	1.480	.530	.360	.510	.004
.353	16.530	1.370	.090	.310	1.280	.000

Chapter 9 - Two-stage work (2 batch and fed-batch vessels operated in tandem).

Section A - Introduction.

Most batch and fed-batch processes, when they are scaled-up from laboratory to pilot and then production scale, require a sequential system. This usually involves using reactors which increase in size by ten-times. By allowing each vessel to grow well, and transferring the contents before stationary growth proceeds, the largest vessel can operate optimally. This would often be run as the final, fed-batch stage, particularly for a process involving the manufacture of **PHB** (or **P(HB/HV)** copolymers). A number of studies have been undertaken, which have proved that multiple-batch culture is superior to single-stage, fed-batch or continuous culture.

Imanaka, Kaeida and Taguchi (1973[94]) examined the production of α-galactosidase from a ***Monascus*** species mold. Using a variety of configurations, two-stage continuous culture, in normal chemostats, decreased the enzyme productivity and specific activity, when compared to single-stage continuous culture. The optimum system used a two-stage chemostat-tubular reactor linked system, with recycle to the first-stage chemostat. The feed streams were split into two, so that specific reactions occured in each stage. Without the recycle, the

productivity was boosted over and above that seen in a single-stage chemostat. With an intermediate reactor, which was a smaller chemostat, the productivity was increased 40%, but the activity was not improved. The best system increased the productivity by some 76%.

Shimizu, et al (1985[95]), grew ***Micrococcus glutamicus***, to study the formation of L-glutamic acid. This is, of course, directly relevant to **PHB** production, and other secondary metabolite systems. In the paper, ***Candida brassicae*** was quoted as a system. Cell productivities increased in the order of fed-batch, continuous and repeated fed-batch culture, as the best of all.

Industrially, **ICI** have used this multiple-stage culture for **BIOPOL** production. The details of this were given in **Chapter 7, Section B.** In this final results chapter, work from four experiments will be detailed. Of these, one was abandoned due to overnight loss of pH control. This led to some cell death and lysis. This, in turn, caused foaming, which rapidly got out of hand. Therefore, detailed information was gained from three experiments. One of these was a comparison between the research strain (**C5**), and the production strain (**TRON**). This experiment nearly led to the rapid cessation of the project, due to probable free butyric acid production - the Department was rendered "cheesy" for one week!

The technique for multiple-stage culturing, or any form of scale-up, is very important, in one aspect:- inoculant condition. Unless the contents of one stage is in a suitable condition, it is pointless carrying on with the overall process. As a consequence, weekly inoculated nutrient agar slopes were subcultured late one day. The next day, the new plate was used to "seed" a 250ml shake-flask, containing 100ml of medium. This was grown up overnight, and transferred (50-80ml) early the following day, into the 2l **LH** reactor vessels. These had previously been made ready for cell growth, by allowing the temperature to stabilise at the operating value. The pH was also set for the appropriate level, and aeration was controlled so that at inoculation, the culture medium would be 100% air saturated. Using this careful, straightforward and logical procedure, growth always proceeded well.

It was hoped to demonstrate, in the work of this chapter, the usefulness of a multiple-stage process, in order to optimise the current knowledge on **PHB** production. In these experiments, being only two-stage, **PHB**-free biomass was to be created in the initial stage. This would be used as inoculant for the second stage. The experiments used 2l biomass forming reactors, and a 16l polymer producing vessel (all **LH**). These are shown in the **Appendix Section**, in two photographs, **Figures A6 and A7**. As the impetus behind these studies was to create a

"continuous" or "semi-continuous" (in the spatial sense, not necessarily fermentation) production of PHB, the initial vessel would be refilled and restarted for the next run. The best way to do this would be to use nearly all the initial vessel's contents, leaving a small volume of cells. To this would be added fresh, sterile medium, with which to grow up a good cell density once again. It was also obvious, that to create a near-continuous production process, more than one production vessel would be required. If this was not the case, then the production would entail a large reaction time, with no product available until the end. In answer to this, during the experiments, the initial 2l was harvested (in the late exponential, to give a good mass of growing cells), and the contents were pumped to the 16l and another 2l vessel. Both second stage vessels were grown up, and fed glucose in the stationary phase, once cell growth had ceased. The initial vessel was not refilled, as would be necessary in a production scheme. The feasibility of running two second-stage production vessels was successfully demonstrated, in a staggered fashion. The staggering was achieved by inoculating one vessel at a time. In an industrial process, this would mean that if operated properly, at any one time, product would be available from a vessel. This would be dependent on a large number of vessels being used, however. In the experiment, only the 16l production vessel was investigated. The possibility of the simple, manually

operated version of an envisaged process, was successfully carried out. With adequate control procedures, an industrial scheme would be run relatively easily, in an automated system.

Section B - Experimental results of multiple-stage fed-batch PHB production.

The first experiment, involving 2 and 16l vessels, was started using techniques described earlier in the chapter. The 2l batch, **PHB**-free biomass formation lasted 12 hours. As in all these experiments, the medium was number 5, as detailed in **Chapter 4, Section B.1, Table 3.** The initial specific growth rate (μ), between 3.5 to 12 hours, was $0.334h^{-1}$, with a population doubling time (t_d) of $2.06h^{-1}$. This was once again worse than the shake-flask experiments. It was fairly good, however, when compared to other batch runs done in the reactor vessels, using medium 5. It is also important to notice that at 12 hours, the culture was still growing exponentially, which was vital for the transfer to the 16l vessel half an hour later. The 16l vessel was seeded, and recording began after 11.5 hours. Sometime during the night, during the initial 11 hours, the alkali pump failed. As a result, the pH was down to 4.8, from 6.8. At 11 hours, it would be expected that the culture would have a good recordable optical density. It was very low, and decreased until 17.5 hours, which was after pH

correction. The vessel was again allowed to continue overnight, and at 35 hours, the cell density, in terms of absorbance, had risen appreciably. However, after 37 hours, the density fell slightly. At this stage, there was considerable foaming, which got more and more difficult to deal with. As some of the cells had obviously perished during the enforced acidification, it was likely that significant cell lysis had taken place. The liberated protein probably accounted for the foaming. At this stage, therefore, the reactor was shutdown. None of the results were put into graph form, consequently.

The second experiment was undertaken, with one 2l vessel supplying two second-stage polymer production vessels, one the 16l reactor, and another 2l vessel. The growth rate measured in the initial 2l vessel, was $0.344h^{-1}$ (although this was gauged from only two readings, done at 9 and 12 hours).

The 16l vessel, once seeded, was allowed to grow up overnight. After 10 hours, the cell density (measured as absorbance) was reasonably good (**Figure 28**, experiment 2). The absorbance had increased by the next sample, 3/4 of an hour later, with a specific growth rate of $0.337h^{-1}$. This is virtually identical to the batch 2l vessel's result. As exponential growth was still proceeding, but was likely to finish shortly afterwards, glucose feeding started (12.5 hours). This immediately

reduced the growth rate, although it could have been due to nitrogen limitation (at 15 hours, NH_4^+ was absent, **Figure 29**, experiment 2). An hour after glucose feeding started, the level of protein peaked at nearly 1.6g/l (**Figure 30**, experiment 2), indicating good first stage exponential growth. The level of glucose, at this stage, was 13.5g/l (**Figure 29**, experiment 2). After 20 hours, the concentration of protein peaked at 1.85g/l.

As the initial glucose **complement** had not run out, when carbon feeding started, the supply of 0.57g glucose/hour/l would not limit the culture. Indeed, the level of glucose was always recordable, even though it was down to a very low level in the later stages of the experiment. After 32 hours, this level apparently stabilised, and the dry cell weight did not significantly increase in the last 10 hours (**Figure 29**, experiment 2). What changed, however, was the **PHB** content, which increased from 55 to over 70%, at these times, respectively (**Figure 32**, experiment 2). The results of this experiment left several unsubstantiated answers, such as the accurate initial specific growth rate of the production vessel. A second, repeat experiment was therefore necessary. However, the end result appeared very satisfactory, a good biomass was achieved, with a very high **PHB** content. The 2l production vessel had a final protein concentration of 0.91g/l, which if the figure for the larger vessel was used, represented 9% of

the biomass. Thus, the cell concentration would be about 11g/l, with 8.3g/l **PHB**.

The next experiment, as the two previous ones, used the research strain, ***Alcaligenes eutrophus*** **H/16 S301/C5**. Once more, one 2l vessel seeded two production vessels, the 16l and a further 2l reactor. No record was made of the initial specific growth rate of the first-stage reactor. However, it was shown how a transfer at 12 hours would preclude the occurrence of stationary phase cells. The 16l vessel was recorded from 0-12 hours, when the initial specific growth rate was $0.26h^{-1}$. An initial 3 hour lag was seen, and the specific growth was apparently worse. The drop in growth rate was associated with a practical planning difficulty. The 16l fed-batch vessel was not ready on time. As a result, the initial 2l vessel was some 22.5 hours old, and well into the stationary phase. A fresh, refrigerated 100ml culture volume was also added, from one of several 250ml shake-flasks, grown up to seed the 2l vessel. Thus, the 16l vessel would have had a proportion of dead cells, which could have lysed and released toxic metabolites. This accounts for the drop in growth rate, although the culture proceeded well later on, to give a good biomass.

After 12 hours, the glucose had decreased from 20 to 6.4g/l. The rate of glucose utilisation was 0.78g/l/hour, during the exponential phase (**Figure 29**, experiment 3).

This is similar to work described in **Chapter 6**, which also detailed how the stationary, **PHB** formation phase consumed glucose at a rate of about 0.6g/l/hour. It was therefore thought that a supply of 0.57g/l/hour, during the feed regime, would cope with this, allowing for the fact that at the start of feeding, there was already excess glucose. In this experiment, however, the residual concentration of glucose was always very low, typically below 1g/l. After 36 hours, the carbon feed stopped, and some eight hours later, at shutdown, the cell dry weight had decreased slightly, from 15.65 to 15.15g/l. The apparent **PHB** concentration had decreased from about 9.4 to 7g/l, representing 60 to 46% of the cell dry weight, respectively (**Figure 32**, experiment 3). The **PHB** results were, however, possibly incorrect, as some spurious results had been evident. This may have been the fault of sample preparation. To conduct further experiments, the glucose feeding should have continued until shutdown, or the cells harvested immediately after glucose ran out

The maximum **PHB** accumulation rates, for both this and the last experiment, occured at 60 and 55% **PHB** storage, at 0.71 and 0.55g/l/hour (**Figure 34**, experiments 2 and 3, respectively). The dissolved oxygen tension (DOT), and stirrer speeds for both followed similar trends. The rpm increased during exponential growth, as the DOT decreased, and vice versa during the stationary, **PHB** storage phase. The maximum and minimum stirrer speeds

were 840-510 and 965-540, respectively. The DOTs ranged between 68-48 and 80-57%, respectively, during the exponential and stationary phases. A total cell count was made in the last experiment, which gave an initial specific growth rate, from 0-12 hours, of $0.243h^{-1}$ (**Figure 31**, experiment 3). This is not too distinct from the absorbance derived figure, even though it has been the case that total cell counts have given widely inaccurate results previously. The maximum cell density reached was $6.367*10^{10}$ cells/ml.

The final fed-batch two-stage experiment was conducted using the production strain, ***Alcaligenes eutrophus*** **H/16 S301 TRON**. This was the only experiment performed with **TRON**, other than the shake-flasks. The initial specific growth rate (μ), using a plot of ln absorbance (660nm) versus time, was $0.321h^{-1}$ (**Figure 28**, experiment 4). This gave a doubling time of 2 hours 10 minutes. This was also using medium 5, and is only 68% of the shake-flask result, using **TRON**, for reasons given earlier. The total cell count measurement gave a growth rate (μ) of $0.292h^{-1}$ (**Figure 31**, experiment 4). The final cell density reached was only $2.235*10^{10}$ cells/ml, just over one third of the previous C5 experiment. The glucose utilisation by **TRON**, was the highest to date, at 1g/l/hour. Suprisingly, however, once glucose feeding began, after 12 hours (when the culture was still growing exponentially) the glucose concentration never went below

9.75g/l (**Figure 29**, experiment 4). The nitrogen apparently ran out somewhere around 11-12 hours (**Figure 29**,experiment 4), and protein production peaked at 1.03g/l (**Figure 30**, experiment 4), which is well below that seen with **C5**. At the end of the exponential growth phase, 4g/l cells were present (**Figure 29**, experiment 4), containing 5% **PHB** (**Figure 32**, experiment 4). This compares with about 7g/l cells and 10% **PHB** in **C5**. In addition, the dry weight increased by only 50% (to 6g/l), and the maximum **PHB** content reached only 18%, before declining back to about 10%. In many respects, the cells themselves were similar to **C5**, in terms of average weight/cell (expressed as approximately $3g/l/10^{10}$ cells, **Figure 33**, experiment 4). Obviously, for such catastrophic losses, some metabolic change must have occured. The most obvious, but not scientifically determined, was the apparent presence of large amounts of free butyric acid. A strong cheesy smell was liberated from the reactor, which had the characteristic smell of butyric acid. Why this **TRON** strain should not produce **PHB**, and secrete the non-hydroxylated monomer itself, is a mystery. The obvious difference to any parallel work, was the CASE award session. Here, **TRON** was fed on glucose and propanoic acid, to stimulate copolymer production. There is no reason why glucose should not have allowed adequate polymer storage in this experiment. Butyric acid, if it was the culprit of the odour, can be used to produce copolymers itself (**Holmes, Wright and Collins**

(1983[36])). The reason for the failure of this experiment, therefore, remains obscure.

Section C - Benefits of two-stage work, as opposed to single-stage culture.

To produce polymers on an industrial scale, which was the underlying aim of this work, required a good cultivation system. The scenario was complicated, in that to produce polymer, firstly polymer-free cells had to be encouraged, to a good density. Then, cell growth had to be halted, and polymer storage promoted by feeding glucose. This is conducive to a fed-batch system. If a batch system was used, then large concentrations of glucose would initially be necessary, with possibly toxic effects on the cells. Continuous culture produces much less biomass, and PHB, at extremely low dilution rates. This would make for a very inefficient system, with a slow recovery. Two-stage batch culture is not appropriate, as the toxicity problem still remains. Two-stage continuous culture is one option, with a small, high dilution rate vessel supplying a large, slow dilution rate polymer production vessel. Once again, using the information gained with earlier results, the system would not really be efficient enough. Two-stage batch and fed-batch, as used in the work for this chapter, seems to be very equitable. The first stage can give good polymer-free biomass, with which to inoculate a

larger production vessel. This would be operated in batch, until the late exponential stage of growth. Fed-batch glucose feeding regimes would then be initiated, to promote high percentages of polymer and cells. In direct comparison between 1 and 2-vessel work, the increase in biomass density was from 12.4 to 15.5%, a 25% increase (ignoring the CASE award results in this instance). The **PHB** formed was also increased, from about 60 to 70%. This was below the maximum that would have been seen, if glucose feeding had proceeded long enough. Theoretically, 80-90% **PHB** storage levels might have been seen.

Of course, this work does not compete with the CASE award results, where 51g/l cells were formed, and up to 80% **PHB**. However, in similar conditions, this laboratory scale-up has indicated that two-stage batch and fed-batch represents a worthwhile development. What is open to question, was whether it is still the best pathway. To produce polymer efficiently, the recovery requires a steady stream of **PHB**-containing cells to treat. To do this, the production vessels have to be several in number, and staggered, so that a constant stream of cells is available for recovery. This would require very careful control of the first-stage vessel, with frequent emptying and refilling stages. The most obvious method to guarantee biomass to production vessels, operating in a cyclical sequential process, would be a first-stage

continuous culture vessel. This would generate biomass constantly, and would require far less "down-time". A secondary first-stage continuous culture vessel could be on standby, if contamination or a malfunction occured. The reasoning described here is now developed in the industrial model, found in **Chapter 10, Section 2.**

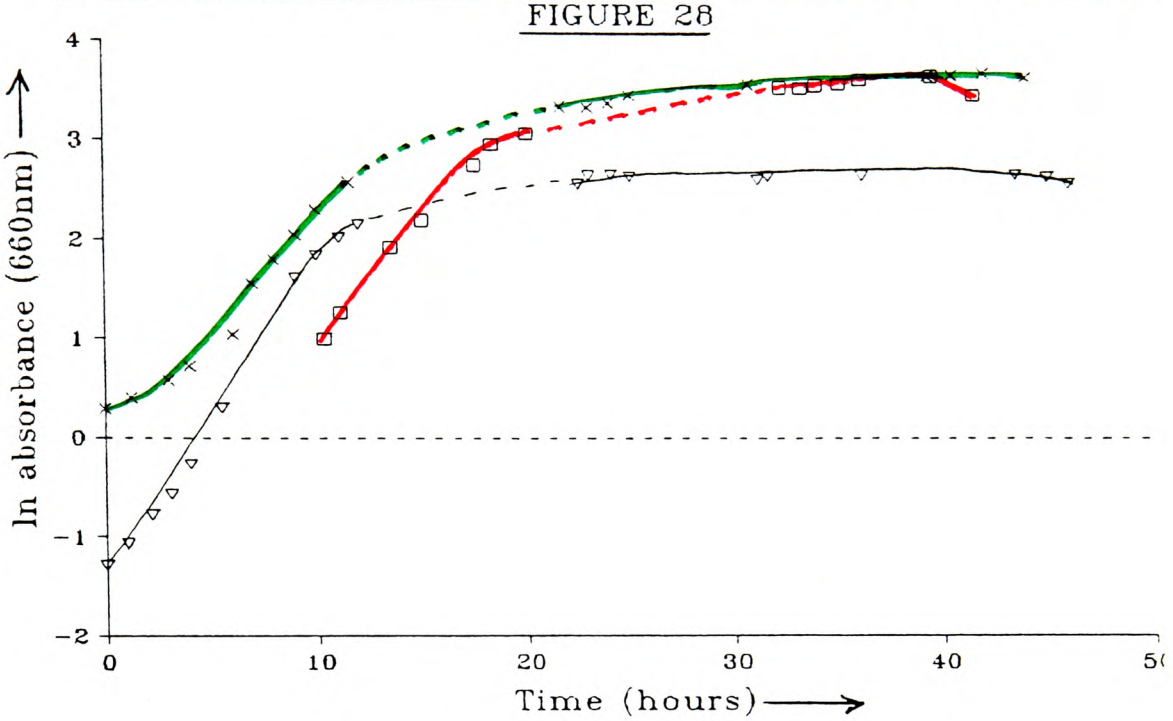
A summary of the results obtained in this chapter's work is shown on the next page, **Table 22. Chapter 10,** the **Conclusions**, examines the aims of the work, after all the experimentation is considered, to see what represents a useful system. This will hopefully be of benefit, even at least as an example of an academic investigation, and hopefully as a potential industrial process.

Table 22.

Experiment	1		2		3		4	
Strain	C5		C5		C5		TRON	
Vessels	A : 2l batch B : 16l fed-batch		A : 2l batch B : 16l fed-batch &2l " "		A : 2l batch B : 16l fed-batch &2l " "		A : 2l batch B : 16l fed-batch &2l " "	
	A	B	A	B	A	B	A	B
Duration (hours)	12.0	37.25	12.0	41.5	22.5	44.0	12.0	46.0
Specific growth rate (μ) (h^{-1}):								
a). absorbance	0.334	-	0.344	0.337	-	0.261	-	0.321
b). cell count	-	-	-	-	-	0.243	-	0.292
Glucose utilisation rate (g/l/h) in exponential phase	-	-	-	-	-	0.78	-	-
Max. total cell count ($*10^{10}$ cells/ml)	-	-	-	-	-	6.367	-	2.235
Cell dry weight (g/l)	-	-	1.909 (end expn)	14.7 (max) (39.5h)	6.9 (end expn)	15.65 (max) (39h)	4.0 (end expn)	6.3 (max) (43.5h)
PHB (g/l)	-	-	-	9.884 (70.1%) (30h) - - - - 6.25 (43.4%) (41.5h)	-	9.421 (60.2%) (39h) - - - - 6.972 (46%) (44h)		0.595 (9.6%) (46h)
(g/l) final protein	-	-	-	1.878	-	2.558	-	0.988
Max. PHB storage rate (g/l/h)	-	-	-	0.71	-	0.55	-	-
at % dry wt.	-	-	-	60.28	-	53.28	-	-

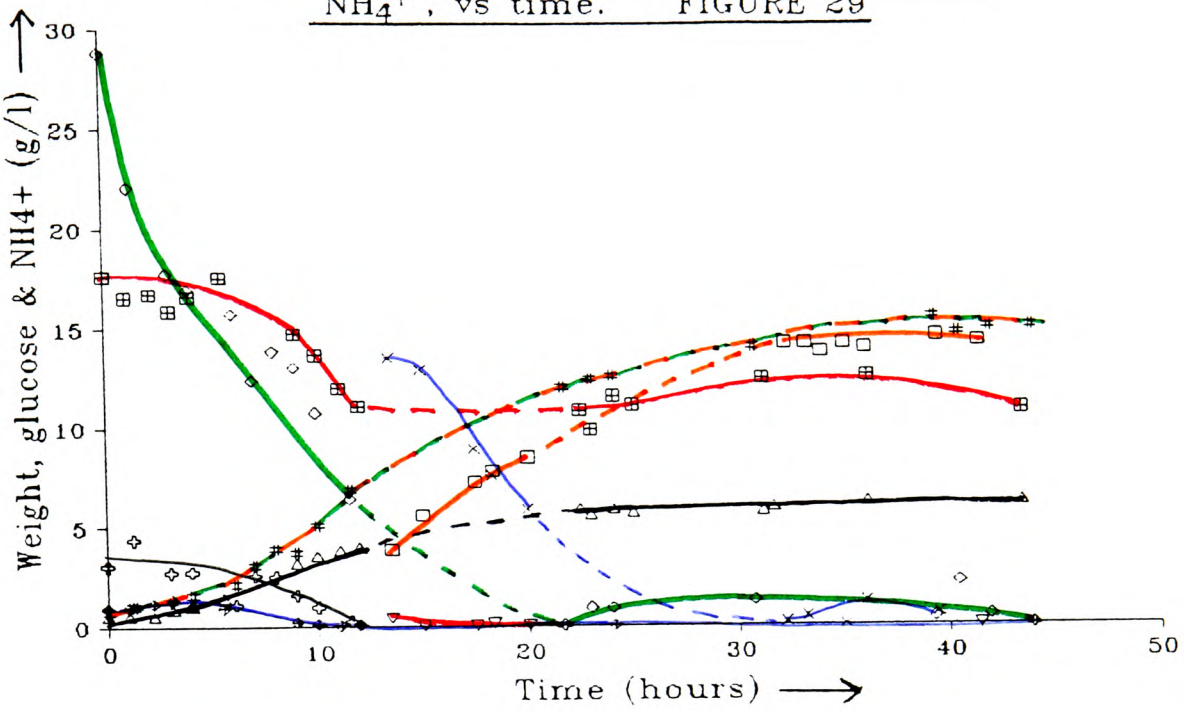
ln absorbance (660nm) vs time (hours)

FIGURE 28



Time (hours)			ln absorbance (660nm)		
10.340	.000	.000	.993	.293	-1.291
11.120	1.250	1.000	1.256	.397	-1.076
13.500	3.000	2.170	1.919	.582	-.781
15.000	4.000	3.080	2.194	.721	-.573
17.500	6.080	4.000	2.740	1.036	-.268
18.333	7.000	5.500	2.949	1.552	.302
20.000	8.000	9.000	3.064	1.793	1.611
32.250	9.000	10.000	3.532	2.054	1.839
33.250	10.000	11.080	3.527	2.307	2.017
34.000	11.580	12.000	3.557	2.572	2.147
35.080	21.667	22.500	3.573	3.330	2.557
36.080	23.000	23.000	3.612	3.322	2.647
39.500	24.000	24.080	3.641	3.371	2.650
41.500	25.000	25.000	3.451	3.444	2.630
	30.750	31.170		3.561	2.610
	39.333	31.670		3.648	2.640
	40.500	36.170		3.657	2.646
	42.000	43.500		3.677	2.654
	44.000	45.000		3.634	2.633
		46.000			2.570
2	3	4	2	3	4

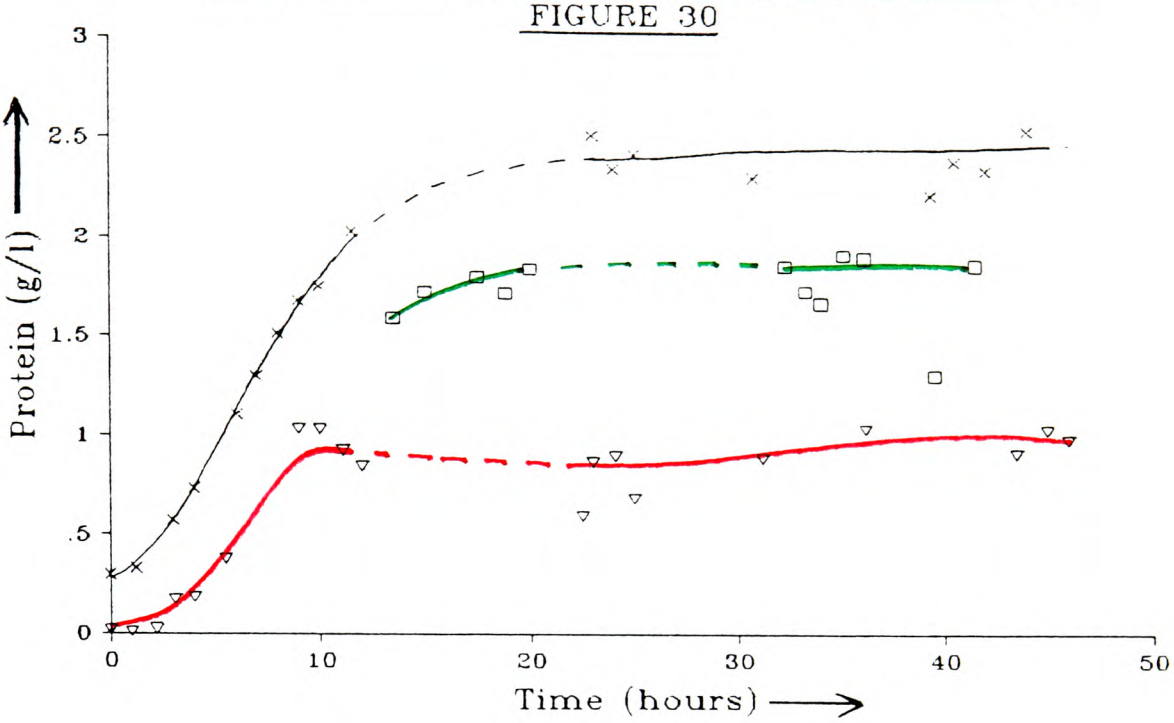
Dry cell weight, glucose & NH_4^+, vs time. FIGURE 29



Time.(hours)			Dry weight	gluc	NH4	Dry weight	gluc	NH4	Dry wt	gluc	NH4
13.500	.000	.000	3.850	13.537	.431	.738	28.806	3.034	.525	17.639	.924
15.000	1.250	1.000	5.600	12.966		1.010	22.068	4.385	.525	16.553	.952
17.500	3.000	2.170	7.300	9.000		1.275	17.775	2.734	.525	16.770	1.157
18.333	4.000	3.080	7.850	7.641	.114	1.575	16.988	2.750	.850	15.901	1.347
20.000	6.080	4.000	8.550	5.902		2.115	15.765	1.099	1.000	16.634	1.044
32.250	7.000	5.500	14.300	.169		3.050	12.423	2.533	1.450	17.585	.917
33.250	8.000	9.000	14.300	.441		3.900	13.890	2.517	3.150	14.787	.228
34.000	9.000	10.000	13.900			3.700	13.075	1.566	3.550	13.727	.108
35.080	10.000	11.080	14.300	.006		5.100	10.793	.965	3.800	12.043	.038
36.080	11.580	12.000	14.100	1.229		6.900	6.446	.240	4.000	11.092	.080
39.500	21.670	22.500	14.700	.659		12.050	.034	.000	5.900	10.902	
41.500	23.000	23.000	14.400			12.480	.903		5.650	9.923	
	24.000	24.080				12.650	.876		5.900	11.608	.000
	30.750	25.000				14.150	1.256		5.750	11.173	
	39.333	31.170				15.650	.414		5.800	12.532	
	40.500	31.670				14.850	2.207		6.000		
	42.000	36.170				15.100	.496		6.300	12.668	
	44.000	43.500				15.150	.061	.000	6.300	10.956	
		45.000							5.900	9.788	
		46.000							6.200	10.059	.000
2	3	4	2	×		3			4		

Protein (g/l) vs Time (hours)

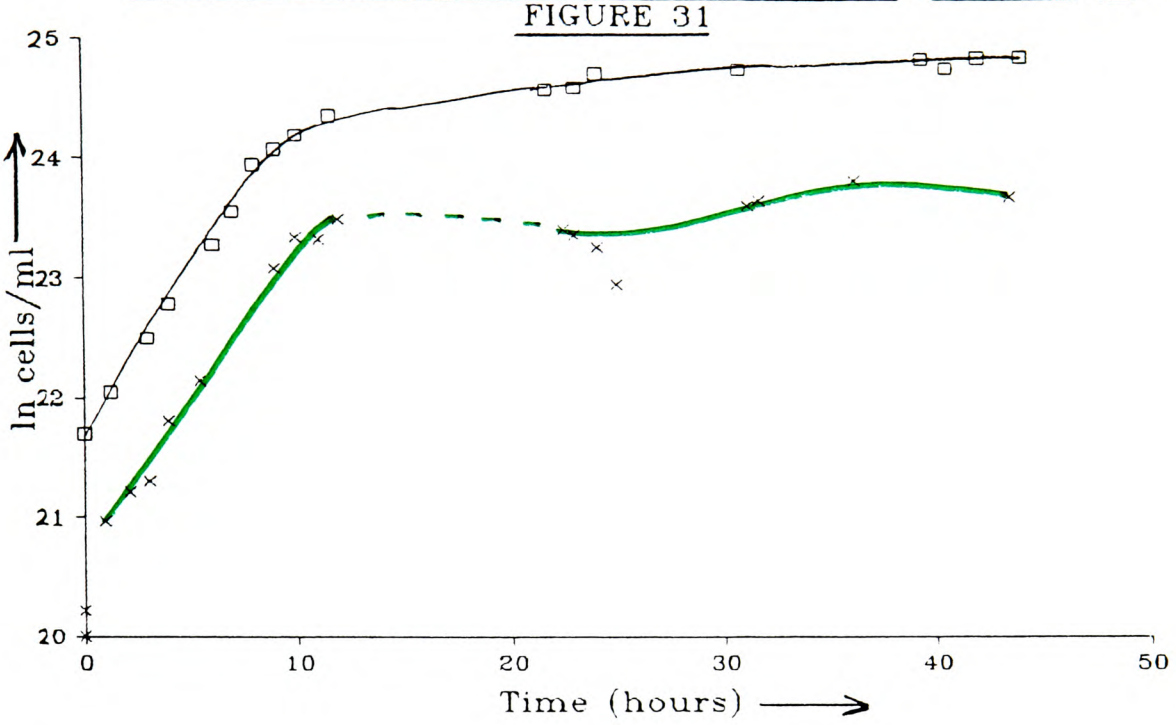
FIGURE 30



Time	(hours)		Protein	(g/l)	
13.500	.000	.000	1.595	.297	.018
15.000	1.250	1.000	1.732	.330	.008
17.500	3.000	2.170	1.809	.572	.022
18.833	4.000	3.080	1.726	.736	.171
20.000	6.080	4.000	1.851	1.108	.183
32.250	7.000	5.500	1.868	1.302	.378
33.250	8.000	9.000	1.743	1.518	1.035
34.000	9.000	10.000	1.680	1.682	1.033
35.080	10.000	11.080	1.924	1.757	.929
36.080	11.580	12.000	1.909	2.034	.847
39.500	23.000	22.500	1.310	2.527	.599
41.500	24.000	23.000	1.878	2.361	.874
	25.000	24.080		2.433	.904
	30.750	25.000		2.319	.688
	39.333	31.170		2.232	.894
	40.500	36.170		2.402	1.043
	42.000	43.500		2.361	.914
	44.000	45.000		2.558	1.037
		46.000			.988
2	3	4	2	3	4

ln total cell count vs time (hours)

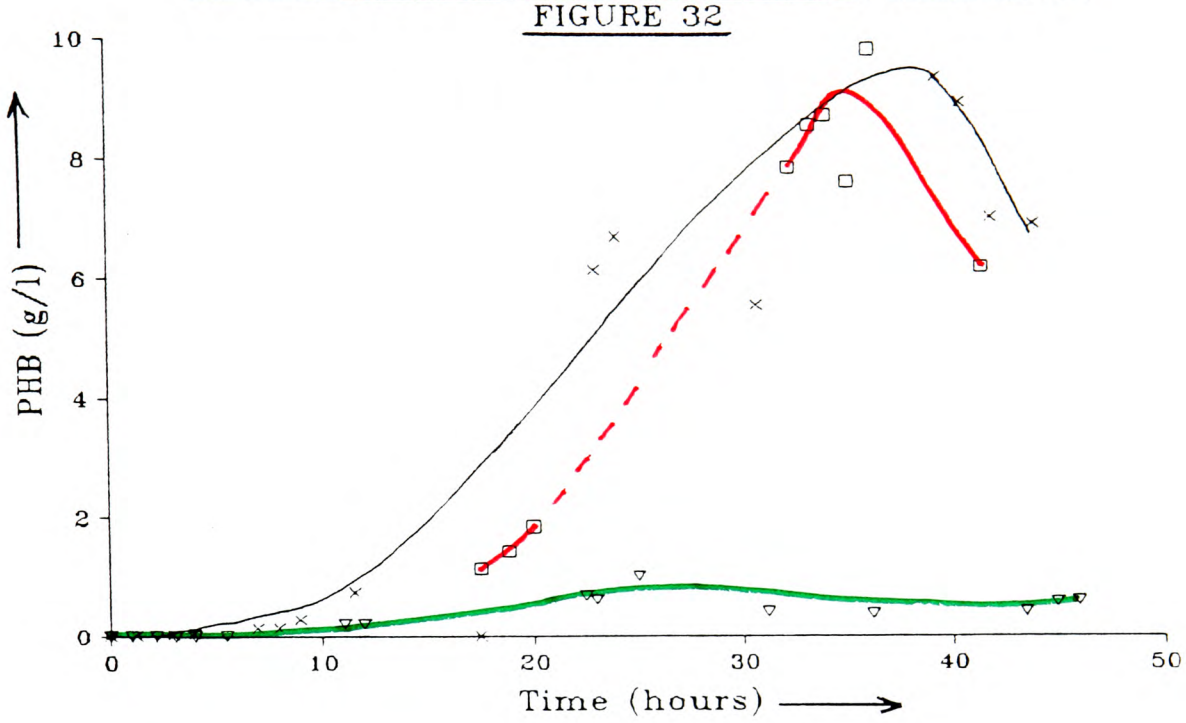
FIGURE 31



Time (hours)		ln cells / ml.	
.000	.000	21.697	20.212
1.250	1.000	22.046	20.966
3.000	2.170	22.500	21.218
4.000	3.080	22.789	21.304
6.080	4.000	23.287	21.813
7.000	5.500	23.564	22.146
8.000	9.000	23.954	23.088
9.000	10.000	24.081	23.345
10.000	11.080	24.196	23.333
11.580	12.000	24.355	23.491
21.670	22.500	24.590	23.425
23.000	23.000	24.607	23.373
24.000	24.080	24.720	23.276
30.750	25.000	24.763	22.963
39.333	31.170	24.860	23.622
40.500	31.670	24.780	23.670
42.000	36.170	24.867	23.832
44.000	43.500	24.877	23.707
	45.000		23.524
	46.000		23.456
3	4	□—□3	×—×4

PHB formation vs time (hours)

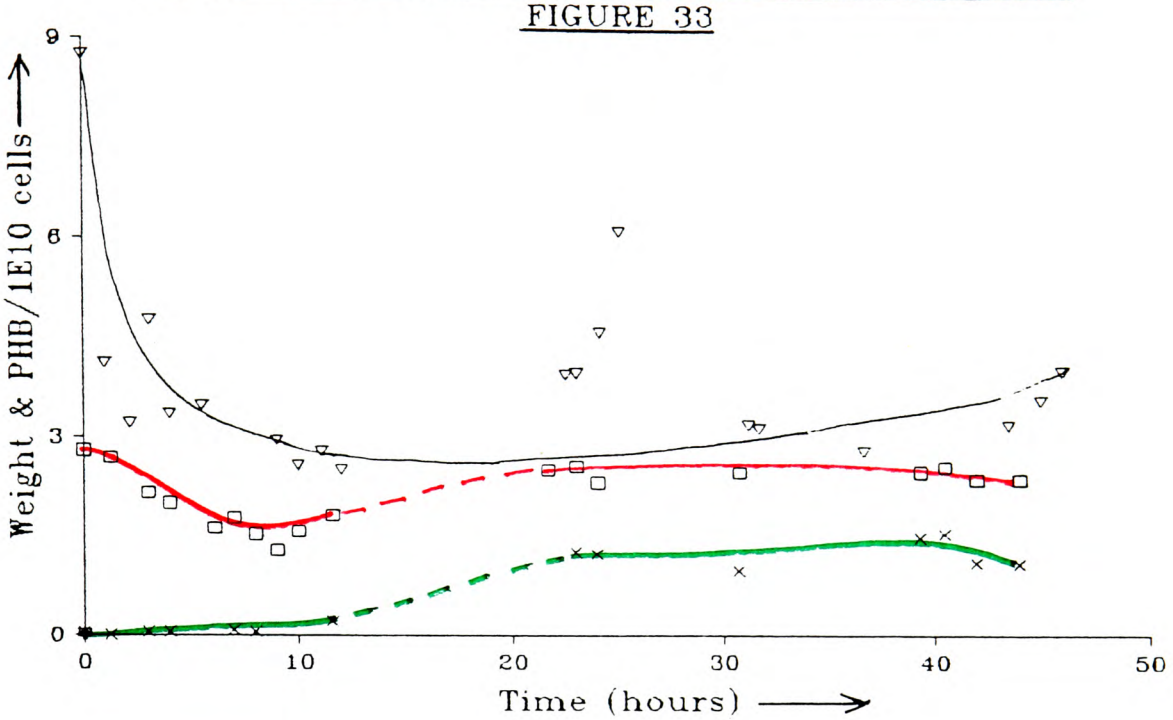
FIGURE 32



Time (hours)			PHB (g/l)		
17.500	.000	.000	1.146	.006	.000
18.833	1.250	1.000	1.444	.002	.000
20.000	3.000	2.170	1.864	.028	.000
32.250	4.000	3.080	7.908	.039	.001
33.250	7.000	4.000	8.623	.139	.000
34.000	8.000	5.500	8.785	.133	.003
35.080	9.000	11.080	7.665	.286	.217
36.080	11.580	12.000	9.884	.735	.204
41.500	23.000	22.500	6.250	6.190	.696
	24.000	23.000		6.742	.616
	30.750	25.000		5.603	1.024
	39.333	31.170		9.421	.412
	40.500	36.170		9.011	.384
	42.000	43.500		7.082	.410
	44.000	45.000		6.972	.578
		46.000			.595
2	3	4	2	3	4

Weight & PHB/1E10 cells vs time

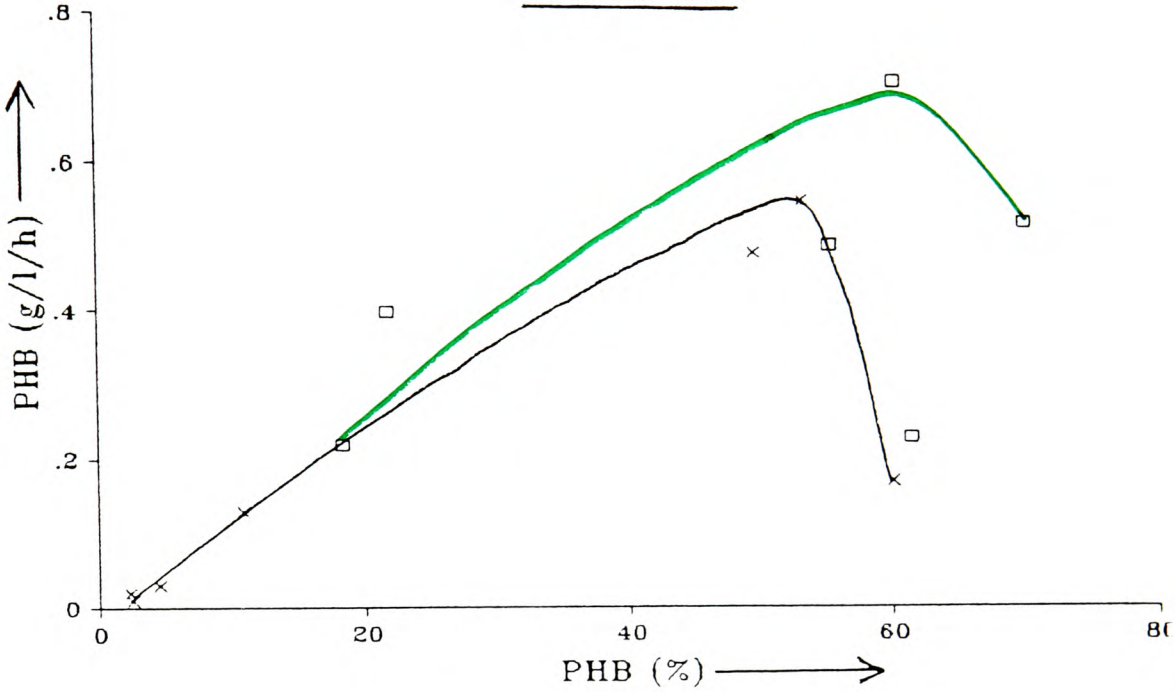
FIGURE 33



Time (hours)		weight/ 1E10 cells	PHB/ 1E10 cells	weight/ 1E10 cells
.000	.000	2.790	.040	8.750
1.250	1.000	2.690	.000	4.120
3.000	2.170	2.160	.050	3.200
4.000	3.080	2.000	.050	4.750
6.080	4.000	1.630		3.360
7.000	5.500	1.780	.080	3.490
8.000	9.000	1.540	.050	2.960
9.000	10.000	1.290		2.580
10.000	11.080	1.580		2.790
11.580	12.000	1.830	.200	2.510
21.667	22.500	2.520		3.960
23.000	23.000	2.570	1.270	3.990
24.000	24.080	2.330	1.240	4.600
30.750	25.000	2.490	.990	6.120
39.333	31.170	2.500	1.500	3.200
40.500	31.667	2.570	1.560	3.150
42.000	36.667	2.380	1.120	2.810
44.000	43.500	2.380	1.090	3.200
	45.000			3.590
	46.000			4.030
3	4	3	3	4

PHB formation (g/l/h) vs % dry weight

FIGURE 34



PHB (%)	PHB (%)	PHB (g/l/h)	
18.340	2.360	.220	.020
21.750	2.530	.400	.010
55.300	4.590	.490	.030
60.280	10.870	.710	.130
61.470	49.600	.230	.480
70.070	53.280	.520	.550
	60.190		.170
2	3	□—□2	×—×3

Chapter 10: Conclusions - Section 1.

Section 1.A - Progress, anecdote to the literature survey.

The last literature search was completed in the summer of 1988. Observation of the **Chemical Abstracts** journals, up until the 8thof May 1989, yielded the following global picture. The reference for all these papers, which have not been considered in this research, are given at the end of this section. They are quoted by the **Chemical Abstract** volume number, followed by the specific abstract number.

The oldest work to come to light now, is that of **Braunegg**. This is a Polish conference paper (1985), published in 1987, which reviews various bacterial **PHB** producers. Information on pharmaceutical drug carrying matrices, and large-scale production of **PHB** by ***Alcaligenes eutrophus*** and ***latus*** is also given.

Also in 1987, several papers were published, on **PHB** production reviews and drug release matrices. The first of these is by **Peoples, et al**, of **MIT**, USA. This concerns a review of **PHB** production by ***Zoogloea ramigera*** and ***Alcaligenes eutrophus***. Possibly for the first time, cloning of **PHB** forming genes was considered. **Walsh, et al**, also of **MIT**, update the review with reference to

polyester biosynthesis. Enzymes involved in microbial biosynthesis are also covered. **Shimizu, et al** (Japan), focus on **PHB** production using methanol as the carbon supply, in their review.

Korsatko, et al (**Graz**, Austria), published two papers on **PHB's** use, as sustained drug release materials. In 1988, **Kubota, et al** (Japan), published a similar paper.

Two papers were published on analysis of **PHB** containing cells. These were by **Helleur**, and **Groom, et al**, both from Canada. They were concerned with on-line fluorimetry and pyrolysis gas chromatography. The physical chemistry of **PHB** was examined by **Scandola, et al**, from Italy.

Doi, et al, published four papers on copolymer production, including the new monomer **4-hydroxybutyric acid**, incorporated into a **poly 3HB/4HB** polymer. **Doi** also published a review on **PHB** production in ***Alcaligenes eutrophus***. Two similar reviews were forthcoming from **Dawes** and **Haywood, et al**, both from **Hull**, UK.

Other papers in 1988 included **PHB** degradation by a mold, by **McLellan, et al**, from Strathclyde, UK. **PHB** formation in ***Azospirillum*** was investigated by **Bleakley, et al**, from Florida, USA. Specific **PHB** applications and extraction procedures were reported as

two Japanese patents. Additionally, in the **Daily Telegraph**, 2/11/88, p3, was a report on how the Japanese were using chitin (from crustacean shells), as a novel biodegradable plastic. More importantly, the statement went on to say that £32 million is being spent on research into alternative biodegradable plastics, by the Japanese Government. It is eventually planned to mass produce alternative plastics, to be environmentally safe. The implications for this speak for themselves.

Two papers were specifically concerned with cloning **PHB**-forming genes into ***Escherichia coli***. The implications of this are also very important. If ***Escherichia coli*** produced vast quantities of **PHB** (it is stated that 30 and 80% cell dry weights have been achieved), then the market could be greatly increased. The safety considerations, however, probably rule out any immediate short-term mass production. The papers were by **Slater, et al** (USA), and by **Schubert, et al** (FRG).

Three papers have been published in 1989 already. One, by **Barnard, et al**, of Cambridge, UK, details important molecular implications on the in-vivo **PHB** granule, deduced from analysis of live cells by NMR. Two further papers describe the production of **poly-hydroxyalkanoates** (which include **HB** and **HV**), by ***Rhodospirillum rubrum*** and ***Pseudomonas cepacia*** (USA and Canada, respectively).

It is evident, therefore, that the study of **PHB** is steadily being intensified, with several notable groups emerging. With all this work, the whole sphere of the study of **PHB** is being revolutionised. No discussion of this section's references is given, the reader is left to address each on it's merits. The references are:-

Chemical Abstracts, Volume 108 (1-6/88).

3197, 36305, 137792, 220285.

Chemical Abstracts, Volume 109 (7-12/88).

38538, 53165, 55353, 55600, 71987, 74036, 167018, 225247.

Chemical Abstracts, Volume 110 (1/89-8/5/89).

22226, 54253, 55850, 71437, 71521, 93447, 111452, 121263, 121312, 133718, 149883, 155864, 171747 and 171751. Also:-

J.Bact (1988) 170 (10) p4431-4436.

J.Bact (1988) 170 (12) p5837-5847.

Section 1.B - Future work to be considered.

1. Comparison between **ICI Zoladex** and **BIOPOL**, for release of drugs.
2. Use of the **Chem-X** program to work on new and hypothetical monomers, and the influence of polymer properties.
3. Evolutionary study of **PHB** producers - was a "proancestral cell" responsible for all the different types of **PHB** formers seen today?
4. Use of different carbon sources, and copolymer screening with established organisms.
5. Full effect of pH on various stages of production.
6. Different monomer formation, with unusual bacteria, and associated screening.
7. Anaerobic formation of **PHB** (***Clostridium***?).
8. Computer simulation, for experimental design.
9. Air-lift reactors, use and comparison in **PHB** production.
10. Marketing, to see whether the public would pay more for **PHB** products, in light of recent greater environmental awareness. "Biodegradable" plastic bags are available, from **Nelson Packaging**, of Lancashire. This product, "Byoplastic", is a starch lattice, which breaks down to leave very small fragments of plastic, which are supposedly broken by soil fauna and flora. Carrier bags for various firms use this material. **Body Shop** apparently stock only these types.

Section 1.C - Conclusions based on the Introduction.

1. The first thing to bear in mind, by prospective students and their supervisors, upon choosing the research title, is that it is not exclusive. Adjustments to the original aims of the project are sometimes desirable.

2. Microbial production of polymers, as the general title, needs to consider:-

Microbiolgy

Genetics

Process Engineering

Economics

to formulate a successful industrial process. In addition, marketing strategies also have to be considered, as does safety. The product must not be toxic or harmful in any way.

3. Microbial production of **PHB** can be promoted by limiting O_2, N_2, K^+, Mg^{2+}, SO_4^{2-}, Fe^{2+}, Mn^{2+} and phosphate. It is formed to provide the cell with reserves of both carbon and energy.

Section 1.D - Conclusions based on the Literature Survey.

1. To conduct a successful literature search, using the facilities of library computers, a few specific keywords

have to be used. It is possible to limit the search to specific dates, in order to update without duplicating previous searches. Without a careful small selection of keywords, the search becomes unwieldy, and can become very costly - the search is based on a telephone link to one of several search sites, in this case **Rome**!

2. It is very likely, based on the wide distribution of **PHB** accumulators, that a "proancestral cell" may have been responsible for the eventual evolution of the myriad of cells able to synthesise **PHB**. An endosymbiotic relationship probably accounts for ***Tetrahymena*** (a protozoan) being able to do this, to date. The evolution of **PHB** producing cells is, in itself, a possible target for academic research.

3. Several major world groups, for the study of **PHB** production have been established. These include **Professor Edwin Dawes'** group, of **Hull University**, and **ICI Biological Products**, both in the UK. In Europe, **Professor Hans Schlegel**, of **Göttingen University**, West Germany, and **Robert Lafferty**, from **Graz University**, Austria, are the most noticeable. In Japan, **Professor Yoshiharu Doi**, from the **Tokyo Institute, Yokohama**, is a recent source of work. **Elmar Heinzle**, currently at the **ETH University, Zurich**, Switzerland, who worked with Schlegel and Lafferty, has worked on **PHB** simulation. Computer models have been developed, to mimic the system, based on

experimental results. Modelling has an important place in experimental design, as it can pinpoint likely areas, which may be overlooked by conventional methods.

4. The organism ***Alcaligenes latus*** has been put forward, by Laffery's group in Graz, as a superior species. It can store in excess of 60% **PHB** in exponentially growing cells. It is also claimed to be as fast growing and synthesising **PHB**. In one fed-batch experiment, using two linked 15 and 25l vessels, a productivity of 210g/hour polymer was proposed. Successfully attempts to repeat this, in this country have failed, probably as the patent does not give all necessary information.

5. ***Halobacterium mediterranei*** has also been put forward as a novel polymer producer. This extreme halophile can accumulate up to 45% cell dry weight, with much less sterility required. This is due to the salinity, which keeps in check the possibility of contamination. Unfortunately, no mention is made of the cell concentration achieved.

6. In 1980, **ICI** were proposing to create **PHB** with methanol as the carbon source. This year, Japanese workers have reviewed current **PHB** production using methanol utilising organisms. The best production gave a cell density of 206g/l cells, using a methylotroph.

Therefore, ***Alcaligenes*** is not seen as the sole source of **PHB** production, worldwide.

7. The various ***Alcaligenes*** species which can synthesise **PHB** are ***faecalis, ruhlandii, latus, eutrophus and aquamarinus.***

8. In 1976, **ICI** first investigated **PHB** production. Peter Senior and Paul Holmes described how it may come to be used. To date, no commercial product is available, but **ICI's BIOPOL** may well be commercially launched shortly.

9. The study of **PHB** has come a long way, not least in the study of monomers. In addition to **3-hydroxybutyric acid** monomers, there are new descriptions of **4-OH and 5-OH butyric acid**, as well as **5-Hydroxyvaleric acid.** Some of these were characterised by Professor Doi's group, who produced a copolymer of up to 90 mol% **HV** monomers. This has important implications for the **BIOPOL** range. If a broad range of copolymers is produceable, then the process will be more flexible. The copolymer can be formed by feeding cells glucose and various organic acids, such as propionic acid.

10. The market for biopolymers in the 1990s is discussed in a paper from **ICI Biological Products.** The total market would be 700 tonnes of adhesives/coatings, 900 tonnes of fibres, 40000 tonnes of gum, and 2000 tonnes of plastic.

This would be worth in excess of £200 million. It is also stated how ICI have produced **PHB** and copolymers in 50 and $200M^3$ reactors. These would produce about four and sixteen tonnes of **PHB**, respectively, in one fed-batch run.

11. Very detailed media characterisation of chemoautotrophically grown ***Alcaligenes eutrophus*** has been carried out. In this, specific minimum saturating concentrations of various minerals were quoted. From this, Cr, Ni and Co were used to supplement the chemoorganotrophic work carried out in this research. Whether this would be of specific benefit is not readily proven. However, the inclusion was done to help long-term experimentation. Careful control of minerals and culture conditions may give rise to very dense cultures, of up to 250g/l. Already, cultures as dense as 200g/l have been demonstrated.

12. A 1974 review gave very detailed information into **PHB** granules. It was stated that they were composed of discrete regions. In work published this year (1989), by researchers at Cambridge, UK, **PHB** granules may be more hydrated and even mobile.

Section 1.E - Conclusions based on the Scope of the work.

1. For industrial **PHB** production, ***Alcaligenes***

eutrophus grown in two-stage continuous to fed-batch culture is the most likely answer.

Section 1.F - Conclusions based on the Materials and methods.

1. To successfully conduct microbial research, the state of the inoculum is vital. Frequent sub-culturing is necessary.

2. For cultivation of ***Alcaligenes eutrophus*** **H/16 S301/C5**, an optimum DOT of 60% is required for growth. Aeration at 0.3vvm is adequate for laboratory work.

3. Measurement of growth can be achieved by various means, depending on the organism. Here, the easiest to use were absorbance readings. On-line mass spectrometry remains one of the best methods of all, though.

4. The graphs presented in this thesis were replotted using **Supercalc 3**. This gave more flexibility than the previous method, especially for final printing.

Section 1.G - Conclusions based on Chapter 5.

1. The first aim of the work was to get a suitable medium. Rather than spend too much time on media formulation, previous media had to be examined. These

were updated with specific data, where available, on individual components.

2. Of the five media initially chosen, the maximum specific growth rate was $0.539h^{-1}$. Subsequent examination of two further media, gave maximum specific growth rates of 0.726 and $0.605h^{-1}$, for the **C5** and **TRON** strains.

3. The provision of additional trace metal Cr, Ni and Co was done to aid long-term fed-batch. In the short-term, exponential growth was not enhanced by this inclusion.

4. Media constituents can be toxic or limiting, if used in the wrong concentration. Ranges have to be devised, which could profoundly affect batch culture. Here, all the nutrient needs to be added initially, instead of constantly in fed-batch or continuous culture.

5. In order to choose a suitable medium, comparative methods are necessary. Student's T test was most satisfactory, and the half chess-board allowed quick comparison, and a ranking system to be devised.

6. Investigation of scale-up problems (from shake-flask to 1l reactors) led to the conclusion that iron precipitation was responsible. This occured with pH control, and was not due to K^+ toxicity, or CO_2 degassing. Use of a 13% CO_2 supplemented air supply gave

a three fold reduction in growth. The optimal level of $Fe_2SO_4.7H_2O$ was 25mg/l. When a 1l reactor was grown without pH control, the growth rate was $0.54h^{-1}$, on medium 5. This was not significantly lower than the shake-flask result. With pH control, a drop in growth rate (compared to previous results) of 40 - 60% was observed.

7. ***Alcaligenes latus*** was quoted as being able to produce up to 60% **PHB**, during exponential growth. It is very difficult to grow, and salt intolerant (no growth occured on normal nutrient agar, with 5% NaCl).

8. Manual pH control of shake-flasks showed tht if pH was kept above 6.3, growth would not suffer.

Section 1.H - Conclusions based on Chapter 6.

1. During batch culture, optical densities and cell counts were recorded. Glucose, NH_4^+, protein and cell weight were recorded.

2. Batch cultures in the 1l vessels allowed for 12 hours exponential growth. Glucose was fed, as small volume, concentrated solutions, at the end of exponential growth, and at intervals during **PHB** storage. Glucose utilisation during growth occured at $0.8g/l.h^{-1}$, and protein formation was equally fast.

3. Polymer storage was initiated by limiting either NH_4^+ (predominantly) or PO_4^{3-}. Extra carbon was required to produce **PHB**, above the 10% normally found in exponentially growing cells. If glucose became limiting, then any **PHB** formed would itself be degraded.

4. Sample collection can pose problems in a small vessel. Vacuum filtration for cell dry weights allowed the use of the filtrate for glucose and ammonia determination. Too much sampling could drain the vessel, expose the heating element and uncover the pH, temperature and DOT probes.

5. For fed-batch experiments, glucose utilisation proceeded at a rate of 0.5-0.6g/l.h^{-1}, during the storage phase.

6. Initially, **PHB** results were gained from gas chromatography. Machine failure terminated it's usage.

7. Additional parameters were developed. As cell counts were frequently done, the average weight and **PHB** concentration of a set number of cells ($1*10^{10}$) could be determined. This clarified what was happening throughout the stages. A graph of storage rate versus % accumulation allowed accurate feeding of glucose, to remove the potential toxicity problem.

Section 1.1 - Conclusions based on Chapter 7.

1. *Alcaligenes eutrophus* **H/16 S301/PRON**, a mutant developed from the production strain, **TRON**, gave significantly higher concentrations of **HV** monomer inclusion, when grown on glucose and propionate. **PRON** was genetically stable, no reverting back to **TRON**. This would enhance the **BIOPOL** range.

2. **TRON** grew well at 40°C, although higher temperatures were inconclusive. This would enable cooling and operational costs to be reduced.

3. Fed-batch duration could be reduced from 72 to 48 hours. **TRON** could be fed glucose and propionate at a faster rate, without apparent toxic effects.

4. Polymer recovery was investigated fully. The current technique is suitable for large-scale production of polymer. For bench-scale, the lower temperature and concentration detergent worked very well. Heat-shocking was only necessary for production runs, to facilitate operation of the centrifuges, and reduce loss.

5. For polymer analysis, five techniques were examined. Gel-permetaion chromatography and melt-flow index were qualitative, differential scanning calorimetry could be used either qualitatively or quantitatively. Nuclear

magnetic resonance and high performance liquid chromatography were used as the best quantitative test for mol% **HV** inclusion. Unfortunately, neither gave identical results. **PRON** produced a copolymer with 18-23% **HV**, and **TRON** 10-12% **HV**. The melting points of these were 123^{o}C and 135-139^{o}C, reflecting the high **HV** inclusion (pure **PHB** melts at 170-180^{o}C). Both were high molecular weight polymers ($1*10^{6}$ daltons).

6. The method used to determine polymer content of cells was validated for use with copolymer containing cells. Because of enzyme specificity, copolymers appeared to decrease the content. A calibration curve was devised, so that when the **HV** content was accurately known, the results could be adjusted properly to take account of copolymers.

7. A production run was attended. 7 mol% **HV** copolymer was produced in a $50M^{3}$ vessel, supplied by 0.5 and $5M^{3}$ vessels. 110g/l cells were produced, with 73% polymer, over 70 hours in the final fed-batch stage reactor. This gave 5.5 tonnes of cells, and 4 tonnes of polymer. The cell and polymer yields were 0.42 and 0.31 respectively.

Section 1.J - Conclusions based on Chapter 8.

1. A continual system would be ideal for an industrial process. For this reason, continuous culture was

examined, and to compare with batch and fed-batch. A considerable advantage would be the reduction in down time and non-operational work, and hense cutting costs.

2. For polymer production, there would be two linked chemostats, the first 25% the volume of the second. The first one would consequently run at a higher dilution rate, and provide polymer-free cells. The second, at a dilution rate four times lower, would suit polymer production.

3. Foam control is very important in continuous culture. Washout could occur, due to volume reduction and dilution rate increase. A foam probe and timed dosage system would be necessary for proper operation.

4. Various automation techniques are most suitable for continuous culture. Use of a spectrophotometer and a flow-through cell, linked in a sterile loop to the reactor, can provide automatic readings. If linked to a computer, growth rate could be calculated automatically. The limitations would be the culture density, spectrophotometer linearity and chosen wavelength. A fraction collector can be used to get as much data as possible out of the chemostat. Providing the cells can be kept stable, neither growing or lysing (formaldehyde and refrigeration), this is very useful.

5. Continuous is not really suitable for polymer production. Only 20-25% polymer was accumulated, and at very low dilution rates ($0.1h^{-1}$). A critical dilution rate of $0.35-0.4h^{-1}$ was gauged, coinciding with the best growth rate seen in batch and fed-batch work, using medium 5 and with pH control. Washout was not achieved.

6. The major conclusion of this chapter, is that continuous culture could be used to produce biomass for a second-stage fed-batch polymer producing reactor vessel.

Section 1.K - Conclusions based on Chapter 9.

1. To scale-up batch and fed-batch processes, sequential reactors are used, rising in volume by a factor of ten.

2. For polymer production, using two-stage culture, a 2l reactor fed 2 and 16l vessels, which were staggered. This would mean a constant stream of finished product would be available, whilst other vessels were still running. The first-stage biomass producing vessel would be either totally or partially emptied, refilled and regrown. Several biomass producing reactors could be used, to create a constant stream of biomass for the second stage.

3. The first and second stage reactors grew equally well, during exponential growth. Glucose feeding, during the fed-batch second stage, should have been prolonged past

36 hours, or the culture should have been harvested at this point (to prevent loss of polymer already formed). The highest cell density achieved was realsied using this system (except for the **CASE** award work), giving 15.65g/l cells. Polymer accumulation, up until glucose ran out, was very good, as was the accumulation rate, peaking again at 60%. **TRON** did not grow well in this system.

4. To increase productivity, the order of effectiveness is single-stage fed-batch, then continuous and finally multiple-stage (or repeated) fed-batch culture. In order to remove excess downtime, and facilitate a semi-continuous system, continuous culture (for biomass) would be run together with several staggered fed-batch production vessels. This would enable product recovery to take place continually. This theme will be developed in **Section 2**, where a model will be devised.

Section 2 - Process Modelling

Section 2.A - Formulation of a limited-scale industrial model based on the conclusions of the work.

The following model was created due to results and conclusions arrived at throughout the research. Basically the system comprises a two-stage process:

(i) **Continuous culture vessel** - the biomass production reactor which is linked to and feeds stage (ii).

(ii) **Fed-batch reactor** - final **PHB** production vessel. This would be operated firstly batchwise, when biomass from stage (i) would be pumped onto uninoculated medium in reactor (ii). The cells would be allowed to grow up to a suitable density, without the formation of **PHB**, and with sufficient nutrients to allow exponential growth. After about 12 hours of such growth, nutrient limitation (nitrogen starvation) would proceed. The fed-batch operation would now start, with approximately 50 hours of carbon feeding. Depending on whether pure **PHB** or a variety of copolymers of **PHB/PHV** were required, the C-source could be glucose or glucose/propionic acid mixtures. After the fed-batch stage and harvesting were finished, the reactor would be turned around for another cycle.

Details of the system.

For the basic model, the production vessel, stage (ii), would be a **50M^3** reactor vessel. This would require 1 continuous culture vessel, the size of which is determined by the results of continuous culture experiments (see Chapter 9). From this, it is seen that a dilution rate (D) of $0.25h^{-1}$ would be eminently suitable. This would produce a supply of biomass at around 4g/l cell density. Using a $1/10^{th}$ scale reactor, relative to stage (ii), there would be a **5M^3** continuous culture chemostat, filled to **4M^3** working volume. For a dilution rate of $0.25h^{-1}$, this would require a flow rate of **1M^3** sterile medium/hour, and produce **1M^3** biomass/hour. To supply the **50M^3** production reactor vessel, with a working volume of **40M^3**, it needs **4M^3** biomass as a "seed" for the reaction. The **4M^3** inoculation would therefore be provided in 4 hours, at the specified flow rate, from the chemostat.

To provide a suitable reservoir for fresh medium, to supply the production vessel (stage (ii)), and chemostat (stage (i)), a **100M^3** medium hold tank would be required. This would allow several cycles of the main production stage vessel before needing refilling. All 3 vessels would be made of **304L grade Stainless Steel**, and rated as pressure vessels capable of withstanding 150 lbs pressure. This is to allow repeated sterilisation. In

addition, the system would require a harvest storage tank, again of **100M^3**. This would be a non-pressurised **304L Stainless Steel** vesel. This would be fitted with a cyclical sprayer unit, which ensures homogenisation of the harvest material. The tank would have a small amount of concentrated acid at the bottom, to bring the final pH to 5.0. This prevents any further reaction, or degradation by cellular enzymes. Two further vessels are required, a reserve tank to supply the chemostat whilst the large medium tank is refilled, and a concentrated carbon feed tank. Both of these would be constructed in pressurisable **304L Stainless.**

To operate the above system properly, in order to maximise use of the continuous production of **PHB**-free biomass, the following time schedule is used:

Process Details	Time (hours) needed for process	Cumulative Time (hours)
a)Assume production vessel (stage (ii)) is clean, empty and sterile.	0	0
b)Fill above vessel with **36M^3** medium, from **100M^3** medium hold tank.	1	1

Process Details	Time (hours) needed for process	Cumulative Time (hours)
c)Seed production vessel with inoculum from chemostat, vessel (i)	4	5
d)Exponential batch growth in production vessel (stage (ii)).	12	17
e)Once process d) complete, cells at 5g/l in $40M^3$, start fed-batch regime (C-feed).	50	67
f)Harvest; drop into acid hold tank	1	68
g)Wash, drain, sterilise and redrain	2-4	72
h)Go back to a). Total time (hours) for 1 cycle:		72

Thus the complete cycle takes 3 days (72 hours). It is therefore seen that to use the chemostat output most efficiently, more than 1 production vessel is required. This also provides a suitable degree of flexibility, allowing copolymer production, and guards against the dangers of reactors going "down". If it takes about 3 hours to clean and sterilise a vessel, and about 1 hour to refill it with medium, then the number of vessels required is 72/4, **18 vessels**. This provides a complete system.

Key to the following page's diagram

Reactor 1: A $5M^3$ **reserve medium tank** used to supply

Reactor 2: whilst **Tank 1** replenished.

Reactor 2: A $5M^3$ **chemostat**, $1M^3$/hr steady-state production of **PHB**-free biomass. Working vol. of $3M^3$, dilution rate of $0.25h^{-1}$

Reactors 3-20: $50M^3$ production vessels. Batch then fed-batch. $40M^3$ working volume, $36M^3$ medium and $4M^3$ inoculum.

Tank 1: $100M^3$ **medium tank**, supplying **Reactors 1-20**.

Tank 2: $100M^3$ **harvest tank**, final product held prior to polymer extraction and recovery

Tank 3: $35M^3$ **Concentrated carbon supply tank**, to allow **PHB** production during fed-batch growth.

Pump 1: **Medium stream pump**, from **tank 1**.

Pump 2: **PHB-free biomass stream pump**, from **Reactor 2**.

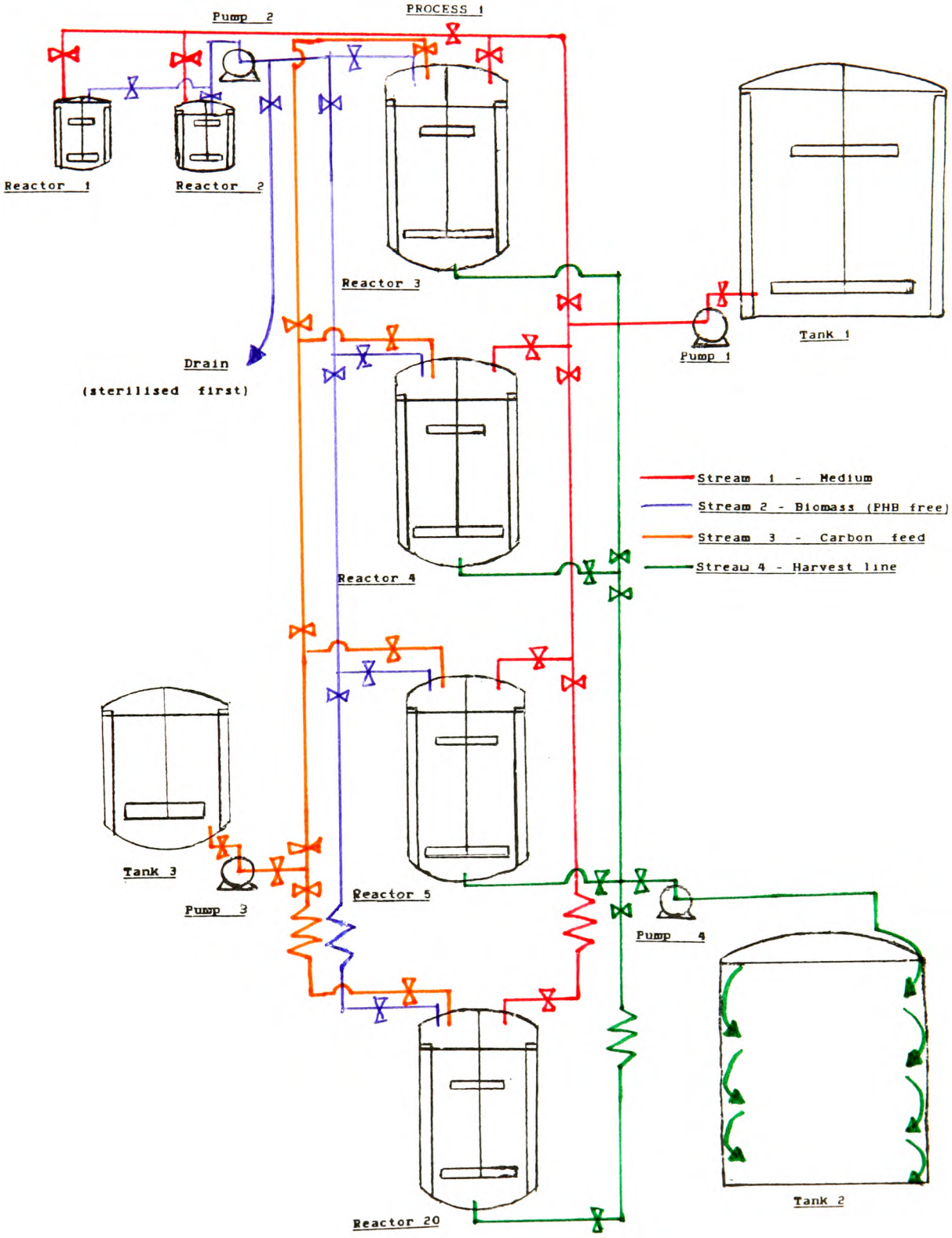
Pump 3: **Carbon feed stream pump**, from **tank 3**.

Pump 4: **Harvest stream**, from **Reactors 1-20**.

Drain: **Residual Biomass**, if a production reactor goes "down", cells are sterilised by steam injection, or the possibility remains for recycling.

><-Principal valves (very much simplified).

Figure 35.


Pump 2
PROCESS 1
Reactor 1
Reactor 2
Reactor 3
Tank 1
Pump 1
Drain
(sterilised first)
Stream 1 - Medium
Stream 2 - Biomass (PHB free)
Stream 3 - Carbon feed
Stream 4 - Harvest line
Reactor 4
Tank 3
Pump 3
Reactor 5
Pump 4
Tank 2
Reactor 20

Section 2.B - Detail of running a system employing 18 production vessels

At start-up of the process, there would be $2x5M^3$ vessels, 1 for continuous culture and 1 for a reserve medium supply tank (to feed the former whilst the main medium supply tank was refilled). There would be $18x50M^3$ vessels, used to produce **PHB**. Two $100M^3$ tanks are needed, 1 for the medium, and the other for the harvesting. A $35M^3$ carbon feed tank is needed for the fed-batch **PHB** accumulation stage.

Once the vessels were clean, the medium hold tank (**Tank 1**) would be filled to $100M^3$, sterilised and made ready for use (pH, temperature, aeration etc. controlled to values needed by the cells). The chemostat (**Reactor 2**) and reserve medium resevoir (**Reactor 1**) are filled to 4 and $5M^3$ respectively. The chemostat (**Reactor 2**) vessel would be seeded, and the cells allowed to grow exponentially to provide a suitable density. Continuous culture would be initiated at a dilution rate of $0.25h^{-1}$ (D). Once steady-state was maintainable, the first $50M^3$ production vessel (**Reactor 3**) starts the production run (at process stage a), 0 hours. After 4 hours the second $50M^3$ production vessel (**Reactor 4**) would be started-up. After 5 hours, **Reactor 1** would pump medium into **Reactor 2** at $1M^3$/hour. **Tank 1** ($100M^3$ medium hold tank) would now be refilled, sterilised and prepared for use. After 8 hours,

Reactor 2 would again be supplied by **Tank** 1, and **Reactor** 1 refilled. The third production vessel (**Reactor** 5) would be filled from 8-9 hours, to $36M^3$. It would be seeded at 9 hours. **Reactor** 6 is filled from 12-13 hours, and seeded at 13 hours. After 9 hours, **Reactor** 1 would again feed **Reactor** 2 whilst **Tank** 1 was replenished. The cycle would continue staggered at 4 hour intervals.

Once the production vessels are seeded completely, the cells are allowed to grow in batch culture. At the end of the exponential growth, fed-batch conditions are initiated. Concentrated carbon is fed for 50 hours. The carbon tank (**Tank** 3), a $35M^3$ vessel, is filled with $33M^3$ of carbon. Where this is glucose, this would be at a strength of $600kg/M^3$, or $0.6Te/M^3$. The flow rate is set to $0.0364M^3$/hour (36.36 l/hour), which over 50 hours is $1.818M^3$ in total. Thus, at the end of the production stage, 1090.8kg glucose supplied to $41.818M^3$ cell material. This is equivalent to about $26kg/M^3$, which over 50 hours equals $0.522kg/M^3$/hour (0.522g/l/hour). During the storage phase, the bacteria assimilate glucose at a rate of about 0.6g/l/hour. Therefore, there should be sufficient supply to enable storage of **PHB** without accumulation of glucose. The rate of supply initially to $40M^3$, due to less volume but the same supply rate, is equal to $0.545kg/M^3$/hour (0.545g/l/hour).

Using the above system, **PHB** production can be

calculated (Te/year).

Operation

Assume a 46-week operational year. There are $40M^3$ cells produced in 72 hours. In addition,there are 18 production vessels. Therefore the production rate of **PHB**-containing cells is:-

$$\frac{40}{72} \times 18 = 10M^3/\text{hour} \times 24 \times 7 \times 46 = 77{,}280M^3/\text{Year}.$$

The cell concentration would be about $150kg/M^3$, of which 75% would be **PHB** ($112.5kg/M^3$). Thus:-

$$\frac{112.5 \times 77{,}280kg\ \textbf{PHB}/\text{Year}}{1000} = \text{Tons (Te) } \textbf{PHB}/\text{Year}.$$

= 8,694,000kg **PHB**/Year, =8,694 Te **PHB**/Year. For calculations, this will be assumed to equal 9,000 Te **PHB**.

To formulate an actual process model based on production capacity versus cost, a projected market capacity (Te **PHB**) has to be stated. This will be set at 500 Te **PHB**/Year. To produce a flexible model, the range between 50-5000 Te/Year will be examined.

If the 500 Te **PHB**/Year production level is considered, the previous system employed 18 x $50M^3$

vessels. Now the system has to consider the use of 18 vessels of a proportionately smaller size. The reduction in size is 500/9000 = $1/18^{th}$ the size. Put into a mathematical equation, this gives.

A + 2B + C + D + E + F = **Product/process cost estimation (PCE).**

A is the **PHB** production vessel reactor size.

To work this out, the number of vessels and their tonnage capacity is needed.

A = a1 x a2/a3

a1 is the number of **PHB** production reactors, here 18.

a2 is the reactor size, ie 18 vessels of $50M^3$, 1 vessel of $900M^3$ or 9 vessels of $100M^3$ (or equivalents between).

a3 is the tonnage reduction. 500 Te is $1/18^{th}$ of 9000 Te.

Thus A = 18 x 50/18 = 18 x $2.78M^3$ vessels. This is rounded up to a suitable vessel size, 18 x $3M^3$.

B is the size of both **Reactors 1 and 2**, which is $1/10^{th}$ the size of the production vessels, therefore = $0.3M^3$.

C is the medium supply tank, a pressurisable tank double the size of **A**, therefore = $6M^3$.

D is the harvest tank, a non-pressurisable tank again double the size of **A**, again = $6M^3$.

E is the carbon feed tank, which for 9000 Te is $35M^3$, and therefore it will be 35/18 (due to the tonnage reduction) = $2M^3$.

F are the 4 centrifugal pumps, 1 for each stream.

This now includes all the relevant vessel sizes, and **A, B, C, D, E, and F** can be costed. The total is termed the **Product/Process Cost Estimation** figure, or **PCE**. A **Physical Plant Cost** (**PPC**) figure is formulated by multiplying **PCE** by various cost indices. This is necessary to account for actual building work, piping, instrumentation, etc. These indices are added to give a factor of 4.2 (biological reactors are complex, due to computer control and other instrumentation, hence the larger factor compared to the literature). The **PCE** figure is multiplied by 4.2 to give **PPC. Fixed Capital Costing** (**FCC**) is worked out by **PPC** x 1.45. This additional factor allows for design and engineering, contractors fee and contingency costs. To update **FCC** to today's prices, this is multiplied by 1.1. This takes account for inflation. This is necessary as the values used to cost the **PCE** figure, (some of which are taken from **Coulson and Richarson, Chemical Engineering Vol. 6, Design. 1983**) are a few years old.

Ultimately this becomes:- **PCE** x 4.2 = **PPC** x 1.45 = **FCC. FCC** x 1.1 = **FCCi** (**FCC with inflation to 1988 prices**).

Two main figures of interest are arrived at; **PCE** and **FCCi. PCE** is used to assess which configuration is more cost effective for each Tonnage required. How is the the most effective configuration worked out? Firstly, 18 production vessels (and also the ancillary tanks and

vessels) are costed, then 9 and finally 1. In this particular example, 9 double-sized vessels (plus all the additional tanks) were cheaper. 1 vessel was most costly and 18 the next cheapest. Further costing of 10-17 vesseis showed that for 500 Te **PHB**/Year, 10 x $5M^3$ production vessels was the best system. The ancillary vessels were scaled accordingly, **Reactors 1 and 2** were $0.5M^3$, **Tanks 1 and 3** were $10M^3$. The Carbon-feed tank (**Tank 2**) was $2M^3$. In this way, **production versus cost**, of the whole process, can be looked at. This in turn enables a **production versus PHB cost, per Te** figure to be gained. The results of this analysis are summarised into:-

Table 24 - Table of Results (PCE and FCCi calculations for the PHB plant).

Figure 36 - Production costs (FCCi) vs PHB production (Tonnes per annum).

Figure 37 - Cost / Te PHB vs PHB production (Te p.a).

From the figures, the optimum system would produce 2000-2250 Te **PHB**/Year, at a cost of £2700-2900 /Te. If 500 Te **PHB** is produced, the cost per Te is significantly higher, at £4050 /Te. The cost/Te (production versus £/Te, **Figure 37**) is seen to rapidly fall as more **PHB** is produced up until 2000 Te. Above this, the cost/Te **PHB**

starts to rise once more. The Plant costs, however, are linear when plotted against tonnage. The optimum figure for production, leading to cheapest cost/Te, indicates a problem with this costing analysis. The actual cost is probably likely to be 50-100 times higher. Vessel prices are likely to be at fault, although indices could have changed further. These sterilisable vessels (most capable of being regarded as bioreactors) are costed as 304L Stainless steel tanks. The cost/M^3 data only goes up to $10M^3$. Values above are extrapolated off a plot of cost/M^3, done on log paper. These are open to interpretational error. This is especially true of the larger ones, up to $100M^3$. Costing the large tanks gives two extremes of cost, therefore.

Pumps are also costed on a 1 pump/1 stream basis. Limited data availbility forced the use of the same sized pumps (4) for each configuration. Additionally, other small pumps would be required (for pH, antifoam control, etc.). The assumption has been made that they have been calculated into the **FCCi** figure.

However, even with these costing problems, the system configuration analysis is likely to be of use. Hopefully this provides the groundwork for further detailed investigation, if necessary. The story does not end with the **PCE** and **FCC** figures. Eventually **Discounted Cash Flow return** has to be covered. The **DCF return** figure provides

an estimate of break-even point for the project (in terms of cash-flow over a period of years from start-up). Research time constraints have prevented the possibility of going further than **FCCi**.

Finally, in conclusion, it has been determined that for a variety of **PHB** production figures, various configurations are required. An optimum cost/Te of **PHB** has been ascertained, theoretically. In addition, plant costs rise linearly with production increase.

Section 2.C - Conclusions of the model, and the theoretical possibilities of alternatives.

The model proposed could generate sustantial polymer, in a semi-continuous system. In theory, continuous culture should have produced the best system of all. If a $10M^3$ chemostat is considered, which is fed by a $2.5M^3$ chemostat, then the following comparison could be made, if continuous culture was just as productive. The $2.5M^3$ vessel would grow cells, without **PHB** formation. The second vessel would produce **PHB** containing cells, at a dilution rate of $0.1h^{-1}$. This would liberate $1M^3$/hour cells, at a theoretical concentration of $150kg/M^3$. Therefore, 0.15 tonnes of cells/hour would give an annual production of 1600 tonnes of cells, at 870 tonnes of polymer. Thus, if continuous culture was able to produce cell densities as high as fed-batch culture, then to

produce 870 tonnes of polymer, chemostats would be vastly superior. To produce a similar quantity of polymer using the model, would require twelve 6-8M^3 vessels. The most direct comparison, is that the model produces 100 tonnes/year, from one 10M^3 vessel. Using a chemostat, this would be 8-9 times more efficient.

A further comparison would be polymer production using ***Alcaligenes latus***. To produce 100 tonnes of polymer, a single 2M^3 fed-batch production vessel, operated in the same manner as the model, would be required. This is five times better than the model, and would be, in theory, forty times more efficient if grown in a chemostat. Therefore, the model in this chapter provides a very practical way to produce polymer successfully industrially. If chemostats could be run as well as fed-batch culture, the situaition would be different, as it would be if ***Alcaligenes latus*** was easier to grow. **Finally, therefore, a semi-continuous polymer production scheme is suggested. This combines polymer-free cell production in a small chemostat, and polymer production in larger fed-batch vessels. This is believed to be the best proposition, using current technological knowledge.**

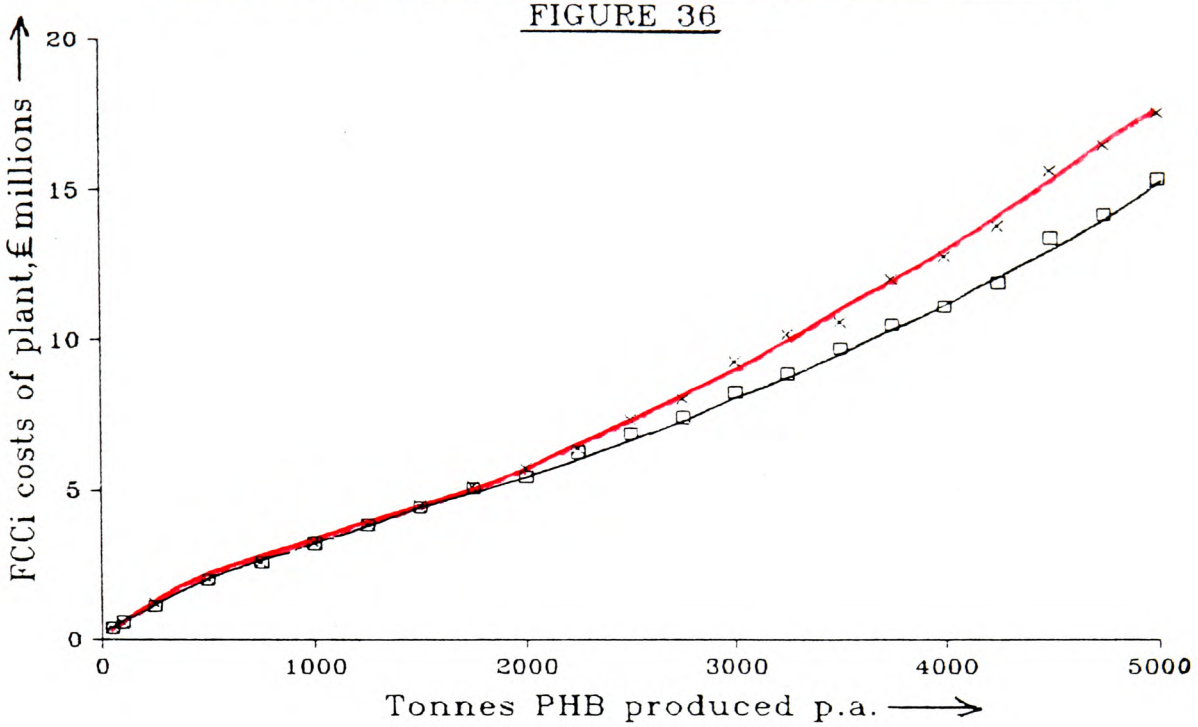
TABLES OF RESULTS, PCE CALCULATIONS FOR PHB PLANT.

Table 24.

PHB production (Te/year)	Production vessel system	PCE figure (£,000s)	FCCi figure, (£,000s)	Optimal price, £/Te PHB (1988)
50	1x 5M^3	58.825	394.07	£7880/Te
100	1x10M^3	86.275	577.96	5780
250	1x25M^3	164.625	1102.825	4410
		-180.525	-1209.34	-4840
500	10x 5M^3	302.225	2024.605	4050
750	12x 6M^3	387.2	2593.85	3460
		-387.95	-2598.88	-3465
1000	12x 8M^3	479.05	3209.155	3210
		-485.3	-3251.025	-3250
1250	15x 8M^3	569.7	3816.42	3050
		-575.95	-3858.29	-3090
1500	18x 8M^3	661.7	4432.73	2955
		-667.95	-4474.6	-2980
1750	17x10M^3	756.05	5064.78	2890
		-766.05	-5131.77	-2930
2000	15x13M^3	815.5	5463.035	2730
		-857.5	-5744.39	-2870
2250	18x12M^3	937.55	6280.65	2790
		-952.55	-6381.13	-2835
2500	18x14M^3	1033	6920.07	2770
		-1105	-7402.395	-2960
2750	18x15M^3	1114	7462.685	2710
		-1207.5	-8089.04	-2940
3000	18x17M^3	1241	8313.46	2770
		-1393	-9331.71	-3110
3250	18x18M^3	1331	8916.37	2790
		-1528	-10236.072	-3150
3500	18x19M^3	1460	9780.54	2790
		-1591	-10658.11	-3045
3750	18x21M^3	1575	10550.925	2810
		-1803	-12078.3	-3220
4000	18x22M^3	1669	11180.63	2795
		-1916	-12835.28	-3210
4250	18x23M^3	1787.5	11974.46	2820
		-2072	-13880.33	-3265
4500	18x25M^3	2011	13471.69	2990
		-2342	-15689.06	-3485
4750	18x26M^3	2125	14235.375	3000
		-2470	-16546.53	-3485
5000	18x27M^3	2299	15401	3080
		-2630	-17618.37	-3520

Cost of plant vs PHB production

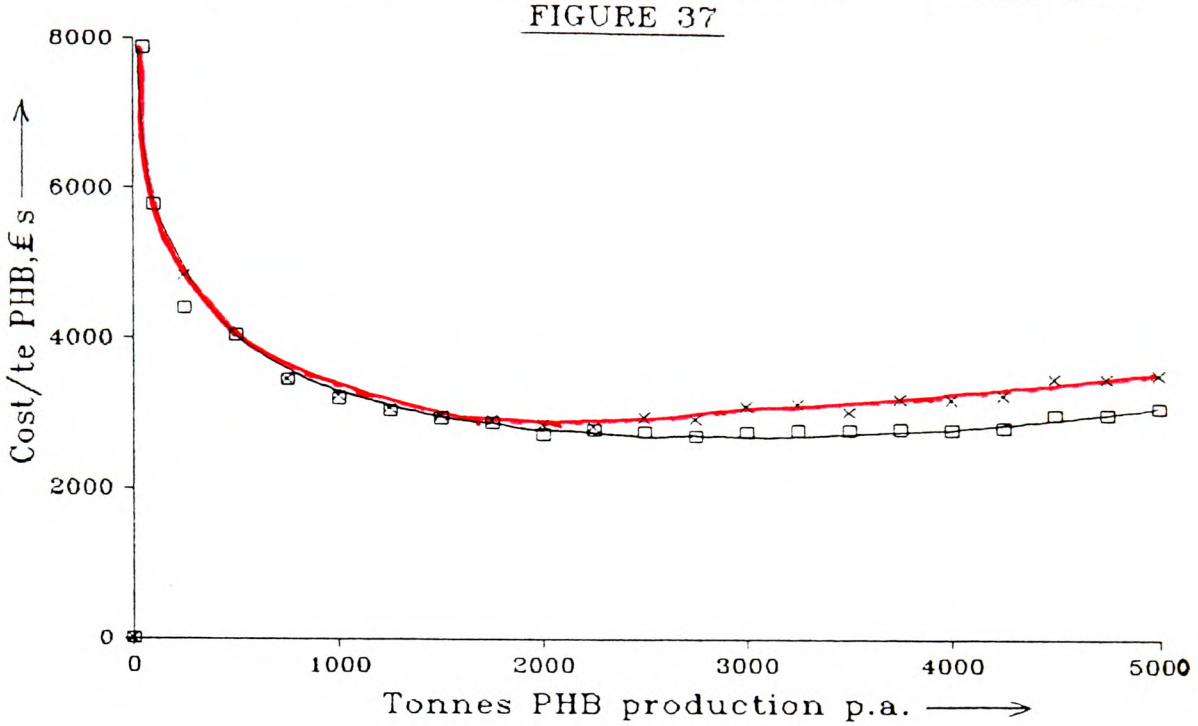
FIGURE 36



Tonnes PHB p.a.	Min. cost	Max. cost
50	.39	.39
100	.58	.58
250	1.10	1.21
500	2.02	2.02
750	2.59	2.60
1,000	3.21	3.25
1,250	3.82	3.86
1,500	4.43	4.47
1,750	5.06	5.13
2,000	5.46	5.74
2,250	6.28	6.38
2,500	6.92	7.40
2,750	7.46	8.09
3,000	8.31	9.33
3,250	8.92	10.24
3,500	9.78	10.66
3,750	10.55	12.08
4,000	11.18	12.84
4,250	11.97	13.88
4,500	13.47	15.69
4,750	14.23	16.55
5,000	15.40	17.62

Cost of PHB vs PHB production p.a.

FIGURE 37



Tonnes PHB p.a.	Min. cost	Max. cost
50	7,880	
100	5,780	
250	4,410	4,840
500	4,050	
750	3,460	3,465
1,000	3,210	3,250
1,250	3,050	3,090
1,500	2,955	2,980
1,750	2,890	2,930
2,000	2,730	2,870
2,250	2,790	2,835
2,500	2,770	2,960
2,750	2,710	2,940
3,000	2,770	3,110
3,250	2,790	3,150
3,500	2,790	3,045
3,750	2,810	3,220
4,000	2,795	3,210
4,250	2,820	3,265
4,500	2,990	3,485
4,750	3,000	3,485
5,000	3,080	3,520

References

How to use these references. In the text, the **Author(s), (year and reference numbers)** are given. The references below are listed as they are used, and not alphabetically by author, or chronologically by date. References are listed with the **journal or book title,** first. This is then followed by a **volume** number, and a part number of the volume (where present). The (year) follows next, with page numbers and **authors/ (and companies, for patents).**

In the **Literature Survey, Chapter 2,** subject sections are described, in which references are reviewed chronologically. However, it may be the case that a later dated reference will appear before an earlier one. This is because papers are used chronologically, according to their submission to the journal. Thus, the first paper would actually be the earliest work, even if it appeared in print later! For patents, the date of the patent/ application publication is given, for the reference. The information would be used based on the earliest submission date. To actually check references, they would be ordered in the form given.

References used in **Chapter 1, Introduction**, first:-

1. **Int.Ind.Biotech.** (6/7 1986) p129-131 - **G.J.Rees and S.E.Montgomery (formerly Underhill)**

2. **Adv.Microbiol.Physiol. 10** (1973) p135-266 - **E.A.Dawes and P.J.Senior**

3. **Ann.Rev.Microbiol. 28** (1974) p167-187 - **J.M.Shively**

4. **Biochem.J. 134** (1973) p225-238 - **P.J.Senior and E.A.Dawes**

5. **Biochem.J. 134** (1973) p239-248 - **V.Oeding and H.G.Schlegel**

6. **J.Chem.Soc., Chem. Commun. 21** (1987) p1635-1636 - **Y.Doi, A.Tamaki, M.Kunioka and K.Soga**

7. **J.Gen.Micro. 95** (1976) p1 34-143 - **I.J.Taylor and C.Anthony**

8. **J.Bacteriol. 116** (1973) p983-993 - **A.C.Emeruma and R.Z.Hawirke**

9. **Makromol.Chem. 146** (1971) p21-32 - **Y.Iwakura, K.Iwata, S.Matsuo, A.Tohara**

10. **Makromol.Chem., Rapid Commun. 8** No.12 (1987) p631-635 - **Y.Doi, A.Tamaki, M.Kunioka and K.Soga**

11. **USA Pat.Appl., 3,107,172** (1963) - **J.N.Baptist and F.X.Werber. (W.R.Grace and Co., New York)**

12. **Cont.Cult.8: Biotechnol, Med. Environ.** (1984) p266-271 - **P.J.Senior**

13. **Phys.Technol. 16** No.1 (1985) p32-36 - **P.A.Holmes**

References used in Chapter 2, Literature Survey, first:-

14. J.Gen.Micro. 19 (1958) p210-222 - R.M.Macrae and J.F.Wilkinson

15. Nature. 189 (1961) p890-892 - J.M.Merrick and M.Doudoroff

16. J.Bacteriol. 90 (1965) p1251-1259 - L.A.Kominek and H.O.Halvorson

17. Biochemistry. 7 (1968) p3676-3681 - R.J.Griebel, Z.Smith and J.M.Merrick

18. Biochem.J. 128 (1972) p1129-1201 - P.J.Senior, G.A.Beech, G.F.Ritchie and E.A.Dawes

19. J.Gen.Micro. 97 (1976) p303-312 - F.A.Jackson and E.A.Dawes

20. J.Gen.Micro. 102 (1977) p61-68 - A.C.Ward, B.I.Rowley and E.A.Dawes

21. J.Gen.Micro. 110 (1979) p393-400 - I.S.Carter and E.A.Dawes

22. Arch.Microbiol. 119 (1978) p231-235 - A.M.Cook and H.G.Schlegel

23. Eur.J.Appl.Microbiol.Biotechnol. 7 No.1 (1979) p1-10 - B.Sonnleitner, E.Heinzle, G.Braunegg and R.M.Lafferty

24. Eur.J.Appl.Microbiol.Biotechnol. 11 (1980) p8-16 - E.Heinzle and R.M.Lafferty

25. J.Gen.Micro. 128 (1982) p2937-2943 - I.Nur, Y.Okon and Y.Henis

26. Israeli J. of Bot. 31 (1982) p221-227 - I.Nur, Y.Okon and Y.Henis

27. Biotech.Bioeng. 26 (1984) p764-770 - R.S.Siegel and D.F.Ollis

28. Acta.Biotechnol. 5 No.4 (1985) p339-345 - G.Braunegg and B.Bogensburger

29. J.Bacteriol. 95 (1968) p1798-1803 - H.Stockdale, D.W.Ribbons and E.A.Dawes

30. J.Osaka; City Med.Cent. 33 No.1 (1983) p15-26 - T.Kitase

31. Can.J.Microbiol. 31 No.7 (1985) p608-613 - S.Tal and Y.Okon

32. Appl.Environ.Microbiol. 51 No.1 (1986) p214-216 - R.Fernandez-Castillo, V.F.Rodrigues, R.J.Gonzales and B.F.Ruis

33. Abstr.Ann.Meet.Amer.Soc.Microbiol. 86 No.0 (1986) p178 - D.W.Martin and J.R.Steward

34. Eur.Pat.Appl. No.0015669 (1980) - K.A.Powell, B.A.Collinson and K.R.Richardson (ICI, UK)

35. Eur.Pat.Appl. No.0046344 (1982) - L.Hughes and K.R.Richardson (ICI, UK)

36. Eur.Pat.Appl. No.0069497 (1983) - P.A.Holmes, L.F.Wright and S.H.Collins (ICI, UK)

37. Eur.Pat.Appl. No.0114086 (1984) - K.R.Richardson (ICI, UK)

38. Biotechnol. Lett. 6 No.12 (1984) P825-829 - G.Braunegg and L.Korneti

39. Eur.Pat.Appl. No.0144017 (1985) - R.M.Lafferty and G.Braunegg (Chemie Linz, Austria)

40. Eur.Pat.Appl. No.0149744 (1985) - R.M.Lafferty and

G.Braunegg (Chemie Linz, Austria)

41. Appl.Microbiol.Biotechnol. 23 No.5 (1986) p322-329 - T.Suzuki, T.Yamane and S.Shimizu

42. Tib.Tech. No.5 (9/87) p246-250 - D.Byrom

43. ICI Document (13/9/87) - D.J.Southgate

44. Nature. 191 (1961) p463-465 - H.G.Schlegel, G.Gottschalk and R.Bartha

45. Arch.Microbiol. 71 (1970) p283-294 - H.G.Schlegel, R.M.Lafferty and I.Krauss

46. Appl.Environ.Microbiol. 32 No.4 (1976) p585-591 - R. & A.C.Repaske

47. Appl.Environ.Microbiol. 32 No.4 (1976) p592-597 - R.Repaske and R.Mayer

48. USA Pat.Appl. No.4138291 (1979) - R.M.Lafferty (Agroferm, Switzerland)

49. J.Gen.Micro. 115 No.1 (1979) p185-192 - C.G.Friedrich, B.Bowien and B.Friedrich

50. J.Gen.Micro. 122 (1980) p69-78 - C.G. & B.Friedrich and B.Bowien

51. Kobunshi 16 No.188 (1967) p1185-1191 - N.Tsukagoshi and K.Arima

52. Biochem. J. 102 No.3 (1967) p33 - E.A.Dawes

53. Analytical Biochem. 52 (1973) p607-613 - A.C.Ward and E.A.Dawes

54. Eur.J.Appl.Micro. 1 No.3 (1975) p233-237 - R.R.Jüttner, R.M.Lafferty and H.J.Knackmuss

55. Eur.J.Appl.Microbiol.Biotechnol. 6 No.1 (1978) p28-37 - G.Braunegg, B.Sonnleitner and R.M.Lafferty

56. Appl.Environ.Microbiol. 43 (1982) p1208-1209 - K.W.Nickerson

57. Biotech.Bioeng. 26 No.8 (1984) p982-987 - F.Srienc, B.Arnold and J.E.Bailey

58. Arch.Microbiol. 143 No.2 (1985) p178-184 - C.Pedrios-Alio, J.Mas and R.Guerro

59. ICI Biological Products, method description (23/10/85) - R.Mann

60. ICI Biological Products, method description (3/4/86) - L.V.M.Johnson

61. Macromolecules 19 (1986) p2860-2864 - Y.Doi, M.Kunioka, Y.Nakamura and K.Soga

62. USA Patent No. 4140741 (1979) - R.M.Lafferty and E.Heinzle (Agroferm, Switzerland)

63. Eur.Pat.Appl. No.0036699 (1981) - P.A.Holmes and L.F.Wright (ICI, UK)

64. Eur.Pat.Appl. No.0015123 (1980) - P.A.Holmes, L.F.Wright, B.Alderson and P.J.Senior (ICI, UK)

65. Eur.Pat.Appl. No.0046017 (1982) - J.Walker, J.R.Whitton and B.Alderson (ICI, UK)

66. Eur.Pat.Appl. No.0046335 (1982) - P.A.Holmes and E.Jones (ICI, UK)

67. Eur.Pat.document, unknown number (1984) - G.B.Lim and P.A.Holmes (ICI, UK)

68. ICI Biological Products, method description (3/6/87) - D.J.Southgate

69. Eur.Pat.Appl. unknown number, the official version of 67 above.

70. ICI Biological Products report (1983) - P.A.Holmes, K.R.Richardson, I.R.Hill and E.A.Drew

71. ICI Biological Products report (1984) - T.E.Robson and P.A.Holmes

72. Personal communication (1988) - C.Rostron (School of Health Sciences, The Liverpool Polytechnic)

73. Pharm.Acta.Helv. 60 (1985) p162-? - J.Rak, J.L.Ford, C.Rostron and V.Walters

74. Polymer Preprints 27 No.1 (1986) p431-432 - D.C.Tunc

75. ICI Biological Products report (1983) - P.A.Holmes

76. Eur.Pat.Appl. No.0052946 (1982) - T.J.Henman and P.A.Holmes (ICI, UK)

77. ICI Biological Products report (1983) - P.J.Barham and H.H.Wills (ICI, UK and Bristol University)

78. ICI Biological Products report (1983) - P.J.Barham, A.Keller, E.Otun and P.A.Holmes (ICI, UK and Bristol University)

79. Macromolecules 19 (1986) p2865-2871 - S.Bloembergen, D.A.Holden, G.K.Hamer, T.L.Bluhm and R.H.Marchessault

80. J.Gen.Micro. 34 (1964) p441-446 - D.G.Lundgren, R.M.Pfister and J.M.Merrick

81. Pharm. Ind. 45 No.5 (1983) p525-527 - W.Korsatko, B.Wabnegg, G.Braunegg, R.M.Lafferty and F.Strempfl

82. Pharm. Ind. 45 No.10 (1983) p1004-1007 - W.Korsatko, B.Wabnegg, G.Braunegg, R.M.Lafferty and F.Strempfl

83. Eur.Congr.Biotechnol. 1 No.3 (1984) p521-527 - R.M.Lafferty, G.Braunegg, L.Korneti, F.Strempfl, B.Bogensburger, W.Korsatko and B.Wabnegg

84. "Chemistry in Britain" (12/1987) p1157 - unknown author

References used in Chapter 4, Materials and Methods, first:-

85. Personal communication (9/1988) - O.Hbarak (Chemie Linz, Austria)

86. Personal communication (1987) - S.E.Montgomery (formerly Underhill) and A.Bitar (University of Bradford)

87. Anal.Biochem. 72 (1976) p248-254 - M.M.Bradford

88. Analytical Chemistry 28 No.3 (1956) M.Dubois, K.A.Gilles, J.K.Hamilton, P.A.Rebers, and F.Smith

89. "Statistical Handbook for non-statisticians" 1st Edn, Publ:- McGraw-Hill (1975) - R.Meddis

90. "Principles of polymer engineering" 1st Edn, Publ:- Oxford Science Publications (1988) - N.G.McCrum, C.P.Buckley and C.B.Bucknall.

References used in Chapter 5, (specific growth rate determination), first:-

91. Personal communication (7/1987) - K.R.Richardson (ICI, UK)

92. Personal communication (10/12/1987) - J.D.Bu'Lock (University of Manchester)

93. Symp. Soc. Gen. Microbiol. 11 (1961) p391-416 - G.Herbert.

References used in **Chapter 9**, two-stage culture, first.

94. J. Ferment. Technol. 51 No.6 (1973) p431-439 - T.Imanaka, T.Kaeida and H.Taguchi.

95. Biotech. Bioeng. 27 (1985) p743-755 - K.shimizu, T.Kobayashi, A.Nagara and M.Matsubara.

Appendix Section.

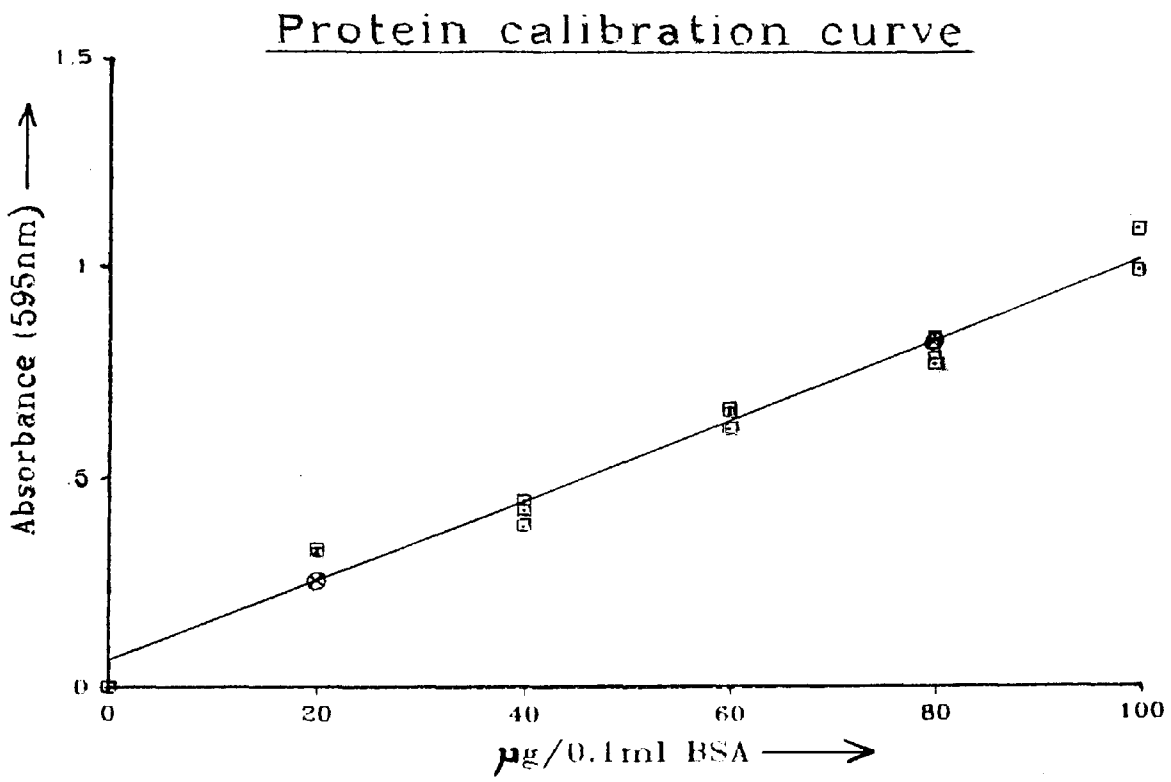
Table A1

Occurence of PHB forming organisms, using Bergey 1974.

Type	Genera
A. Kingdom *Prokaryotae*, Div. 1 - *Cyanobacteria* - Blue/green bacteria	various
B. Kingdom *Prokaryotae* Div. 2 - True bacteria	
1. Phototrophic bacteria (a) Purple non-sulphur bacteria	*Rhodospirillum*, *Rhodopseudomonas*, *Rhodomicrobium*.
(b) Purple sulphur bacteria	*Chromatium*, *Thiocystis*, *Thiosarcina*, *Thiospirillum*, *Thiocapsa*, *Lamprocystis*, *Thiodictyon*, *Amoebobacter*, *Ectothiorhodospira*, *(Chlorogloea?)*.
3. Sheathed bacteria	*Sphaerotilus*, *Leptothrix*.
4. Budding or appendaged bacteria	*Hyphomicrobium*, *Caulobacter*.
6. Spiral and curved bacteria	*Spirillum*.
7. Gram -ve aerobic rods and cocci	*Pseudomonas*, *Zoogloea*, *Azotobacter*, *Beijerinckia*, *Derxia*, *Rhizobium*, *Halobacterium*, *Alcaligenes*, *Methylomonas* *Methylococcus*.
8. Gram -ve facultatively anaerobic rods	*Vibrio*, *Photobacterium*, *Chromobacterium* *Beneckea*.
10. Gram -ve cocci and coccobacilli	*Moraxella*, *Paracoccus*, *Lampropedia*, *Acinetobacter*.
12. Gram -ve chemolithotrophic bacteria	*Nitrobacter*, *Nitrococcus*, *Thiobacillus*.
14. Gram +ve cocci	*Micrococcus*.
15. Gram +ve endospore forming rods and cocci	*Bacillus*, *Clostridium*.
19. *Actinomycetes*, and related organisms	*Actinomycetes*, *Nocardia*, *Streptomyces*
C. Kingdom *Eukaryotae*, subdivision: unicellular protists, microscopic protozoans.	*Tetrahymena*, (possibly due to endosymbiosis of a PHB-forming organism).

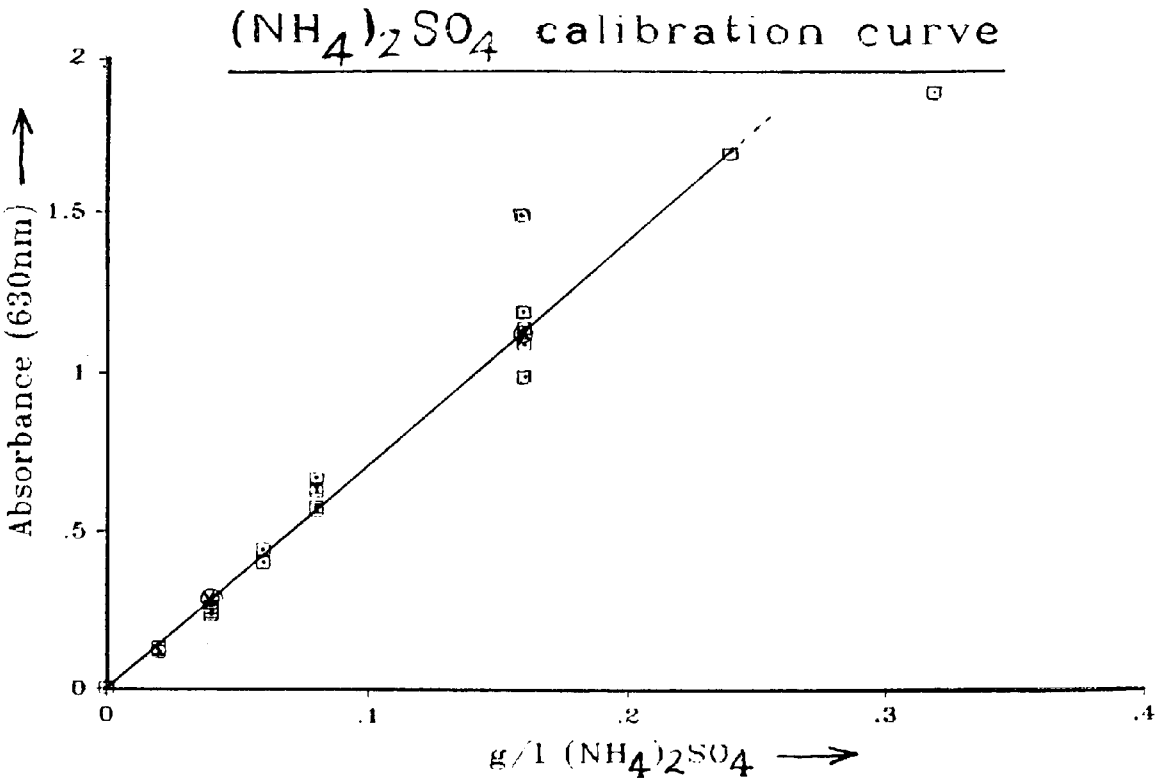
*Refer to the list in the **Introduction** for the current classification, this list is to be used as a comparison.*

Figure A1



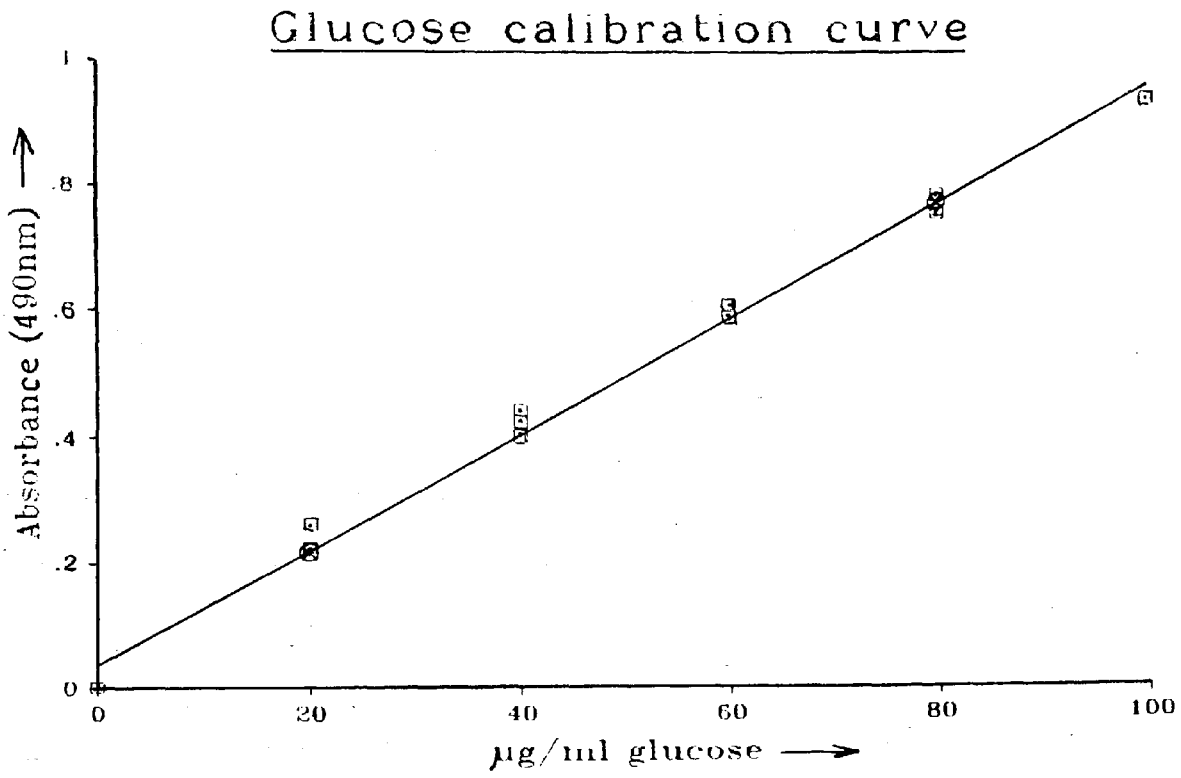
µg/0.1ml BSA	Absorbance (595nm) ⊡	⊗ Best fit values
.000	.000	
20.000	.320	.256
	.330	
40.000	.425	
	.390	
	.450	
60.000	.660	
	.620	
	.670	
80.000	.840	.828
	.775	
	.790	
100.000	1.000	
	1.000	
	1.100	

Figure A2



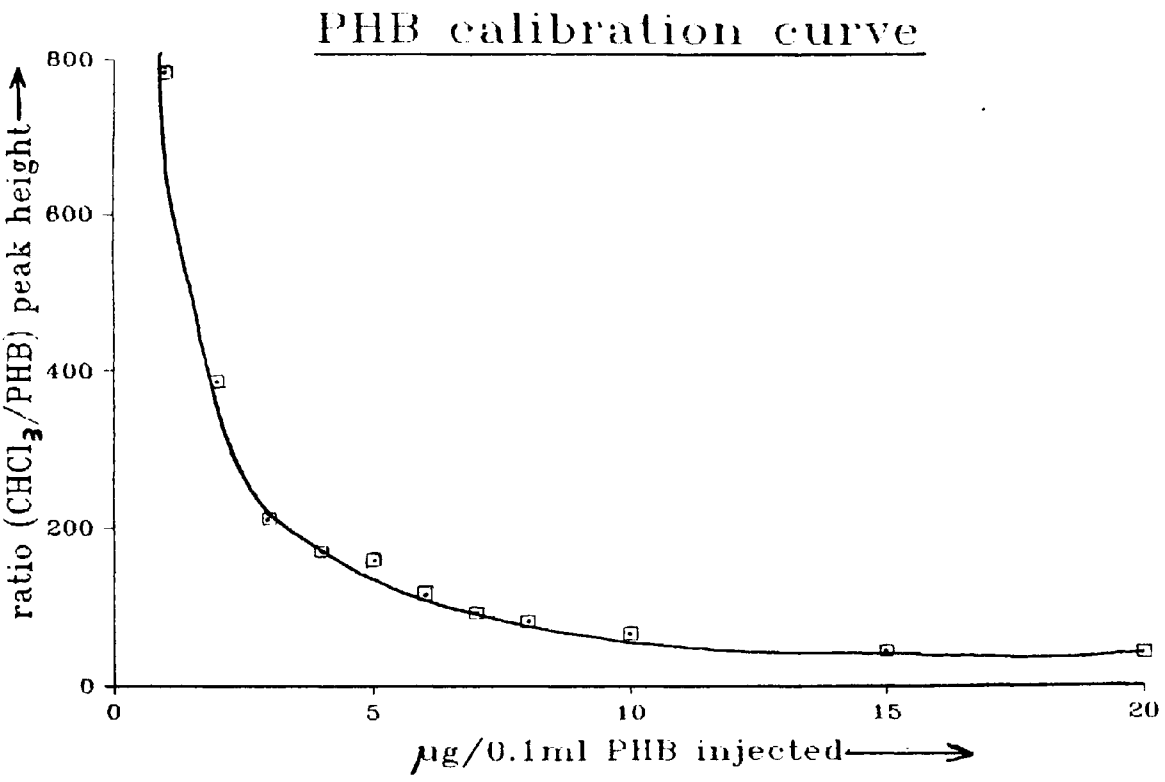
g/l $(NH_4)_2SO_4$	Absorbance (630nm) ⊡	⊗ Best fit values
.000	.000	
.020	.130	
	.115	
	.120	
.040	.245	.286
	.260	
	.235	
.060	.400	
	.405	
	.445	
.080	.580	
	.570	
	.670	
	.630	
	.640	
	.670	
.160	1.100	1.136
	1.000	
	1.100	
	1.500	
	1.200	
	1.150	
.240	1.700	
	1.700	
.320	1.900	

Figure A3



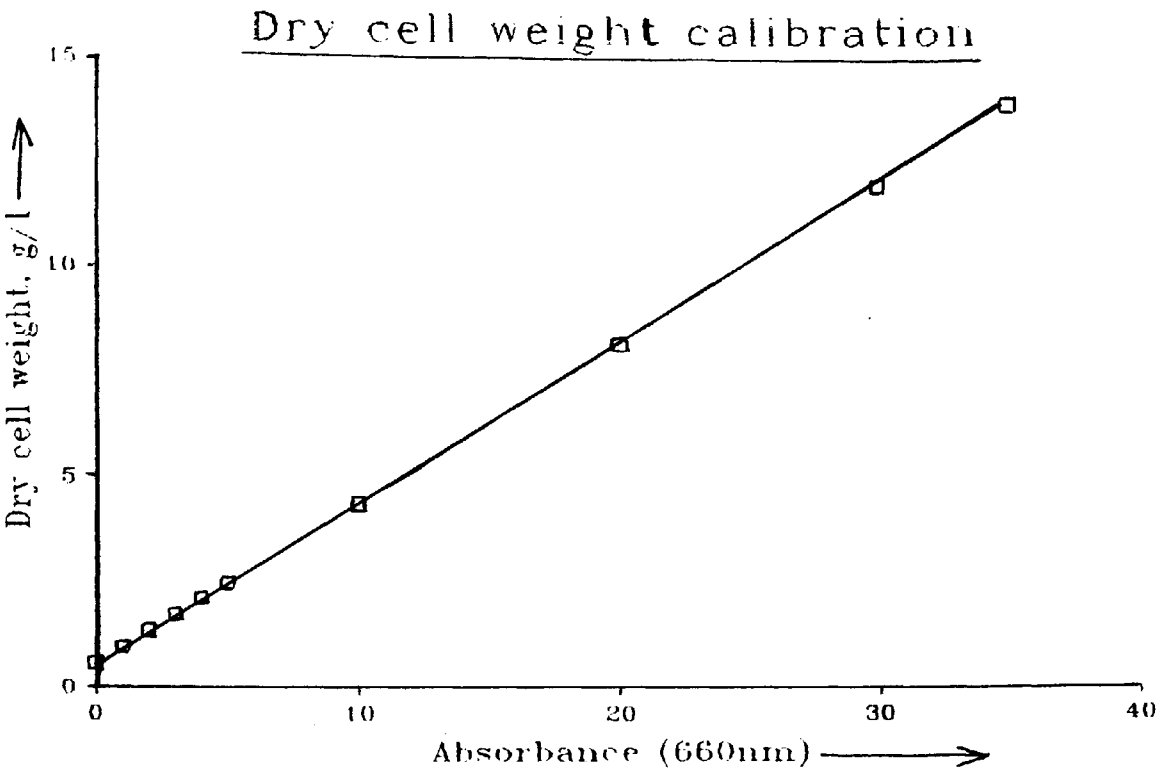
µg/ml glucose	Absorbance (490nm) ◘	⊗Best fit values
0	.000 .000 .000	
20	.263 .213 .225	.217
40	.403 .443 .425	
60	.593 .608 .608	
80	.758 .783 .753	.771
100	.933 .933 .933	

Figure A4



µg/0.1ml PHB injected	ratio ($CHCl_3$/PHB) peak height
1	782.943
2	388.050
3	212.820
4	170.830
5	160.130
6	117.540
7	93.550
8	83.530
10	68.040
15	45.640
20	43.230

Figure A5



Absorbance (660nm)	⊡Dry cell weight, g/l
0	.566
1	.949
2	1.331
3	1.714
4	2.097
5	2.479
10	4.392
20	8.218
30	12.044
35	13.957

Figure A6

Figure A7